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ER Stress in Parkinson's Disease

Miguel de Almeida Santos

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Miguel de Almeida Santos

Dissertação orientada por:

Professora Doutora Maria João Gama

Professora Doutora Elsa Rodrigues

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Neves Carvalho A, Silva-Azevedo C, **A Santos M**, Rodrigues E, Castro-Caldas M, Gama MJ. "Unfolded Protein Response in Parkinson's disease: a new neuroprotective role for Glutathione S-Transferase pi?" 40th FEBS Congress – The Biochemical Basis of Life. July 4-9, 2015 – Berlin, Germany

M.J. NUNES, A.N. CARVALHO, C. SILVA-AZEVEDO, **M.A. SANTOS**, M. CASTRO-CALDAS, E. RODRIGUES, M.J. GAMA. “MPTP-Induced oxidative stress and NRF2-dependent regulation of GSTP gene promotor”. XIV Meeting of the Portuguese Society for Neurosciences 4-5-June 2015, Póvoa de Varzim, Portugal.

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Resumo

O desencadeamento da disfunção do metabolismo proteico nas formas esporádicas da doença de Parkinson (PD) poderá resultar do *stress* oxidativo mediado por espécies reactivas de oxigénio (ROS). O *stress* do reticulo endoplasmático (ER) pode levar à produção de ROS e ao desequilíbrio redox no ER mas a relação exacta entre o *stress* oxidativo e o *stress* do ER está pouco documentada. No entanto, sabe-se que a produção de ROS por neurotoxinas indutoras de parkinsonismo leva a uma rápida acumulação de proteínas oxidadas que por sua vez podem activar a *unfolded protein response* (UPR)

Um potencial mecanismo de defesa celular contra a toxicidade das ROS é a indução da expressão de enzimas de destoxificação de Fase II, nomeadamente a Glutationa S-Transferase Pi (GSTP), pelo factor de transcrição Nrf2. Já demonstrámos que a administração sub-aguda de 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) a ratinhos C57BL/6 induz a expressão da GSTP, e que a degeneração e morte de neurónios dopaminérgicos causadas pelo MPTP ocorre mais precocemente nos ratinhos *knockout* para a GSTP (GSTP KO) do que nos ratinhos *wild type* (wt). Para além disso, o nosso grupo também demonstrou que o ácido tauroursodeoxicólico (TUDCA), um ácido biliar endógeno com propriedades anti-apoptóticas e neuroprotectoras, diminui a morte celular em neurónios dopaminérgicos tratados com MPTP.

Neste estudo, avaliámos os níveis de expressão de marcadores de *stress* do ER no córtex de ratinhos C57/BL6 wt e ratinhos GSTP KO após o tratamento com MPTP. Em paralelo, investigámos também o papel do TUDCA na redução do stress do ER.

Os nossos resultados mostram que neste modelo de PD *in vivo* os ratinhos GSTP KO apresentam um decréscimo nos níveis de expressão de ATF6α e um aumento nos níveis de expressão de IRE1α quando comparados com os ratinho wt. Curiosamente, o efeito mais proeminente da deleção dos genes *Gstp1/2* foi observado nas amostras de ratinhos tratados com MPTP, nas quais os níveis de expressão de Nrf2 se encontram reduzidos de forma significativa nos ratinhos GSTP KO quando comparadas com as amostras

correspondentes de ratinhos wt. Verificámos também que o pré-tratamento com TUDCA modulou os níveis de expressão de diferentes mediadores da UPR após o insulto com MPTP.

Ainda que preliminares, os resultados aqui apresentados mostram que no encéfalo de ratinho, diferentes componentes da UPR apresentam diferentes susceptibilidades ao stress oxidativo induzido pelo MPTP. Essas diferenças estão relacionadas com o genótipo dos ratinhos (wt vs GSTP KO) o que indica que a GSTP possa desempenhar um papel na manutenção do equilíbrio redox no ER.

Abstract

The trigger for dysfunctional protein metabolism, in sporadic Parkinson's disease (PD), may be oxidative stress through damage caused by reactive oxygen species (ROS). The endoplasmic reticulum (ER) stress may trigger ROS production and redox imbalance in the ER but the precise interplay between oxidative stress and ER stress in neurons has been sparsely described. However, generation of ROS by PD triggering neurotoxins leads to a rapid accumulation of oxidized proteins that can activate the unfolded protein response (UPR).

One potential defence against the toxicity of ROS is the up-regulation of phase II detoxification enzymes, namely Glutathione S-Transferase Pi (GSTP), by the Nrf2 transcription factor. We have demonstrated that the sub-acute administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57BL/6 mice induced GSTP expression and that MPTP-induced dopaminergic neuronal degeneration is an earlier event when comparing GSTP null vs wild type (wt) mice. Furthermore, our group has also shown that tauroursodeoxycholic acid (TUDCA), an endogenous bile acid with anti-apoptotic and neuroprotective properties, rescued dopaminergic neurons from MPTP-induced damage.

In the present study, we evaluated the expression levels of ER stress markers in the cortex of C57/BL6 wt mice and GSTP null mice under MPTP-induced oxidative stress. In parallel, we investigated the role of TUDCA in reducing ER stress.

Our results show that in this *in vivo* model of PD GSTP null mice exhibit a decrease in ATF6α expression levels while exhibiting an increase in IRE1α expression levels when compared to the wild type. Interestingly, the most prominent effect of *Gstp1/2* deletion was observed in the MPTP-treated samples, in which Nrf2 expression levels are significantly decreased in GSTP null mice when compared to their wt counterparts. We also observed that pre-treatment with TUDCA modulated the expression levels of the different UPR mediators following the MPTP insult.

Although preliminary, the results present herein show that in the mice brain, different components of the UPR display different susceptibilities to MPTP-oxidative stress. These differences relay on the mice genotype (wt vs Gstp null) indicating that GSTP may have also a role in maintaining the ER redox balance.

List of abbreviations

6-OHDA	6-hydroxydopamine
ARE	Antioxidant-Responsive Element
ASK1	Apoptosis Signal-Regulating Kinase 1
ATP	Adenosine Triphosphate
ATF4	Activating Transcription Factor 4
ATF6α	Activating Transcription Factor 6α
BBB	Blood Brain Barrier
bZIP	Basic leucine zipper
CHOP	C/EBP homologous protein
DA	Dopamine
eIF2	Eukaryotic Translation Initiation Factor
ER	Endoplasmic Reticulum
ERAD	ER-Associated Protein Degradation
GRP78/BIP	Glucose-Related Protein/Binding Immunoglobulin Protein
GSH	Glutathione
GST	Glutathione S-transferase
GSTP	Glutathione S-transferase isoform Pi
GTP	Guanosine Triphosphate
HD	Huntington's disease
IRE1α	Inositol Requiring Enzyme 1α
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
KO	Knockout
MAPK	Mitogen-Activated Protein Kinase
MAO-B	Monoamine oxidase type B
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridinium
MPP^+	1-methyl-4phenylpiridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADH	Nicotinamide adenine dinucleotide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PDP	arkinson's disease
PERK	Double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase
REM	Rapid Eye Movement

RIDD	Regulated IRE1-Dependent Decay
ROS	Reactive Oxygen Species
RNase	Ribonuclease
SN	Substantia Nigra
TRAF2	Tumor necrosis factor Receptor-Associated Factor 2
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
UPR	Unfolded Protein Response
UPS	Ubiquitin Proteasome System
XBP1	X-box binding protein 1
wt	wild type

I. INTRODUCTION AND OBJECTIVES

1. Parkinson's Disease

PD is a chronic and progressive neurodegenerative disorder, characterized by a large number of motor and non-motor features (Jankovic, 2008). Firstly identified in 1817 by James Parkinson, it is the second most common neurodegenerative disease. Its pathophysiological hallmarks are loss or degeneration of dopaminergic neurons in the SN of the midbrain, resulting in a decrease of DA levels, and the development of neuronal LB, abnormal intracytoplasmic aggregates of protein that mostly contain α-synuclein, as well as ubiquitin and phosphorylated neurofilament proteins (Kim *et al*., 2014). DA is a neurotransmitter essential for normal movement, allowing information concerning motor control to be transmitted from the SN to the striatum, which then initiates and controls the ease and balance of movement (Segura-Aguilar *et al*., 2014).

Due to the decrease in DA levels, the symptomatology of PD is characterized by four major cardinal motor symptoms: tremor (e.g. hand tremor at rest), akinesia or bradykinesia (loss of spontaneous movements, facial expression), muscle rigidity and impaired balance and posture (stooping posture) (Hindle, 2010). Furthermore, LB can also occur in multiple areas of the central and peripheral autonomic nervous system, giving rise to a variety of symptoms in addition to the classical PD motor features (Sprenger and Poewe, 2013). There is a big spectrum of non-motor features that PD patients may suffer from, and that may reduce their quality of life, which are not only frequent but also often under-reported by patients and caregivers alike, remaining consequently under-treated (Maass and Reichmann, 2013). These include neurobehavioural disorders like depression, anxiety, apathy, hallucinations, cognitive impairment, impulse disorders (binge eating, pathological gambling) (Chaudhuri *et al*., 2011) and sleep disorders like difficulties with falling asleep, REM sleep behavior disorder and non-REM parasomnias (confusional wandering) (Maas and Reichmann, 2013). Also, PD patients report symptoms like constipation, genitourinary urgency, sensory pain and visual diplopia (Jellinger 2014).

Even though PD symptoms are well characterized, its ultimate causes are still unknown. Recent decades have witnessed a proliferation of medical pharmacological therapies and innovative surgical interventions like deep brain stimulation, but definitive disease modifying therapy is still lacking.

1.1. Epidemiology and risk factors

It is estimated that approximately 1-2% of the population over 65 years suffers from PD, with this figure increasing to 3% to 5% in people 85 years and older (Alves *et al*., 2008). Incidence rates of PD in population-based studies from Europe and the USA range from 8.6 to 19 per 100,000 inhabitants. PD can be sporadic, which comprises the majority of cases, or familial PD, in which patients with the disease report a positive family history. Indeed the highest risk factor for PD is considered to be family history, with monogenic forms, caused by single mutation in dominantly or recessively inherited gene, accounting for 30% of familial PD (Noyce *et al.*, 2012). Other risk factors include pesticide exposure (Wilk *et al*., 2006) and aging.

There were identified 18 specific chromosomal regions, also called chromosomal locus, with a putative link to PD termed PARK (Table 1). However, the causative gene has not yet been identified for all of the loci, with some of them being identified by genetic linkage analysis in large families, and others discovered and established by genome wide association performed on a population level (Klein and Westenberger, 2012). Several genes have been found to be associated with PD (Table 1), namely *SNCA*, which encodes for α-synuclein, the main component of LB, *LRRK2*, a gene that encodes the cytoplasmic protein leucine-rich repeat kinase 2, *PARK2*, the second largest gene in the human genome that encodes for parkin, a protein that functions as an E3 ubiquitin ligase in the process of ubiquitination, *PINK1*, a phosphatase and tension homolog-induced putative kinase 1 that functions in a common pathway with parkin for sensing and selectively eliminating damaged mitochondria from the mitochondrial network, and *DJ-1,* a protein ubiquitously expressed that functions as a cellular sensor of oxidative stress (Klein and

Westenberger, 2012). Each gene has different effects on various aspects of the disease, such as the onset of the disease or the severity of the symptoms.

Table 1 – *PARK*-designated PD-related loci. Adapted from Klein and Westenberger,

Symbol	Gene locus	Disorder	Inheritance	Gene	Status and Remarks
PARK1	4q21-22	EOPD	AD	*SNCA*	Confirmed
PARK2	6q25.2-q27	EOPD	AR	Parkin	Confirmed
PARK3	2p13	Classical PD	AD	Unknown	Unconfirmed; may represent a risk factor; gene not found since first described in 1998
PARK4	4q21-q23	EOPD	AD	*SNCA*	Erroneous locus (identical to PARK1)
PARK5	4p13	Classical PD	AD	*UCHL1*	Unconfirmed (not replicated since described in 1998)
PARK6	1p35-p36	EOPD	AR	*PINK1*	Confirmed
PARK7	1p36	EOPD	AR	*DJ-1*	Confirmed
PARK8	12q12	Classical PD	AD	*LRRK2*	Confirmed; variations in LRRK2 gene include risk-conferring variants and disease-causing mutations
PARK9	1p36	Kufor-Rakeb syndrome, atypical PD with dementia, spasticity, and supranuclear gaze palsy	AD	*ATP13A2*	Confirmed; but complex phenotype that would not be mistaken for early-onset or classical parkinsonism
PARK10	1p32	Classical PD	Risk factor	Unknown	Confirmed susceptibility locus; gene unknown since first described in 2002
PARK11	2q36-27	Late-onset PD	AD	Unknown; not *GIGYF2*	Not independently confirmed; possibly represents a risk factor; gene not found since first described in 2003
PARK12	Xq21-q25	Classical PD	Risk factor	Unknown	Confirmed susceptibility locus; possibly represents a risk factor; gene not found since first described in 2003
PARK13	2p12	Classical PD	AD or risk factor	*HTRA2*	Unconfirmed
PARK14	22q13.1	Early-onset dystonia-parkinsonism	AR	*PLA2G6*	Confirmed
PARK15	22q12-q13	Early-onset parkinsonism-pyramidal syndrome	AR	FBX07	Confirmed
PARK16	1q32	Classical PD	Risk factor	Unknown	Confirmed susceptibility locus
PARK17	16q11.2	Classical PD	AD	*VPS35*	Confirmed
PARK18	3q27.1	Classical PD	AD	*EIF4G1*	Unconfirmed; recently published (Chartier-Harlin *et al*., 2011)

1.2 Pathophysiology of PD

As previously mentioned, PD is characterized by the degeneration of dopaminergic neurons in the SN of the midbrain, which results in a progressive nigrostriatal DA deficiency. It also features the presence of proteinaceous intracellular bodies containing aggregates of α-synuclein. This protein is considered to play a central role in the pathophysiology of PD, as its fibrillar form is known to be a major structural component of LBs in PD and other synucleinopathies (Kim *et al.,* 2014). Accumulation of α-synuclein at the synapse leads to a loss of synaptic proteins and synaptic pruning with loss of

connectivity. *In vitro* studies have shown that α-synuclein aggregates cause a series of secondary processes leading to neuroinflammation, neurodegeneration and cell death, providing compelling evidence that α-synuclein is involved in the pathogenesis of PD (Kim *et al*., 2014). However, it seems that the displacement of α-synuclein monomers from their physiological location in the cells may also contribute to neurodegeneration (Lashuel *et al*., 2013). Furthermore, it is also thought that oxidative damage may play a pertinent role in the aggregation of α-synuclein in PD due to a selective tyrosine nitration of this protein in lesions in PD and other synucleinopathies (Moore *et al*., 2005). The source of the increased oxidative stress is unclear but may include mitochondrial dysfunction, increased DA metabolism that can yield excess hydrogen peroxide and other ROS and impaired antioxidant defense pathways (Jenner 2003). Oxidative stress is considered to compromise the integrity of vulnerable neurons and thus to contribute to neuronal degeneration, and its role in PD will be latter discussed.

1.3 Diagnosis and treatment

Despite decades of research, the diagnosis and subsequent management of PD is hampered by suboptimal methods for detection and prognosis due to the lack of valid diagnostic biomarkers (tests or screening mechanisms) with high sensitivity and specificity that are critically needed for the correct identification of the disease. The definite and accurate diagnosis of PD can only be done through *post-mortem* neuropathological examination of brain tissue from PD patients. During lifetime, the diagnosis of PD relies on clinical criteria, based chiefly in the presence of parkinsonian symptoms. This proves to be a challenging task given the fact that the classic PD symptoms can be present in other neurodegenerative disorders. Careful history taking and astute physical assessment coupled with initial medical therapy are necessary to distinguish PD from other diseases or from secondary parkinsonism due to provoking drugs, toxins, infections and neurological damage (Beitz 2014).

Although there are no available disease-modifying therapies to alter the underlying neurodegenerative process, symptomatic therapies can improve the patients' quality of life. These pharmacological approaches target chiefly the nigrostriatal dopaminergic pathway and attempt to replenish the DA content in the brain even though they do not prevent the progression of PD. L-DOPA remains as the gold standard because it can readily cross the BBB and is converted to DA through the actions of the enzyme DOPA decarboxylase (Hornykiewicz, 2002). However, L-DOPA is also converted into DA in the peripheral nervous systems, causing undesirable adverse events such as nausea and vomiting. Given these effects, it is standard in clinical practice to co-administer L-DOPA with DOPA decarboxylase inhibitor such as carbidopa that does not cross the BBB, reducing the conversion of L-DOPA to dopamine in the peripheral tissue and therefore increasing L-DOPA delivery to the brain (Nagatsua and Sawadab, 2009). However, long-term L-DOPA treatment is associated with significant complications including involuntary movements, dyskinesias and response fluctuations (Tarazi *et al.*, 2014).

Medication should be initiated when patients experience functional impairment from their symptoms. Initial therapy selection typically depends on patients' specific symptoms and age. If motor symptoms are mild but require therapy, MAO-B inhibitors may be used before moving into more potent treatments such as L-DOPA. These inhibitors can increase synaptic dopamine by blocking its metabolization and are currently approved for the symptomatic treatment of early PD and to reduce off-time in patients with more advanced PD, having demonstrated a small symptomatic benefit and potential disease-modifying effects (Connolly and Lang, 2014). However, these agents have limited use in clinical management due to its low efficacy and possible drug interactions with medications that are contraindicated when taken with MAO inhibitors (Chen, 2011). Several promising new families of drugs are being evaluated for treatment of PD, including adenosine A_{2A} receptor antagonists, neurotrophic factors, glutamate antagonists and transdermal nicotine (Payami and Factor, 2014). However, none of these has been approved for general use

and often the reason is insufficient efficacy. In summary, current treatments for PD do not provide adequate neuroprotection and have limited efficacy, being incapable of slowing the progression of this disease and being associated with adverse motor and non-motor side effects. Accordingly, there is an urgent need to develop novel pharmacotherapies that are superior to the current ones, with improved efficacy, safety and long-term maintenance.

1.4 Models of PD

Currently, there is a plethora of animal models that mimic different aspects related to the pathology of PD. All of these experimental models can be categorized into two main groups: toxin-induced models and genetic models. None of the currently available models copy the phenotype of PD, mainly because they lack some specific neuropathological and/or behavioral feature of this disease, with each model having certain advantages and shortcomings. In addition to the classical motor abnormalities observed in PD, animal models are increasingly used to study non-motor symptoms (Campos *et al.*, 2013). Both toxin-based and genetic models are suitable for studying these non-motor symptoms that are increasingly recognized as relevant in disease-state.

1.4.1. Toxin-induced models of PD

Toxin-based models of parkinsonism have been widely used and have yielded a wealth of insight into PD neuropathogenesis while also providing disease models in which to define putative pharmacological targets and to test potential therapies. These models have continued to evolve and have been used to study PD for more than half a century, starting with the 6-OHDA model of parkinsonism, introduced in the late 1950's (Mendez and Finn, 1975). The structure of this selective catecholaminergic neurotoxin is very similar to that of DA, but an additional hydroxyl group on 6-OHDA renders it specifically toxic to dopaminergic neurons. Much of the biochemical, physiological and behavioral effects of nigral dopaminergic neuron loss and striatal DA depletion has been

collected from the 6-OHDA model of parkinsonism, with oxidative stress being widely regarded as the primary mechanism through which this molecule exerts its effects (Martinez and Greenamyre, 2012).

The molecule MPTP was first recognized as a neurotoxin when a group of intravenous heroin users developed an acute version of parkinsonism that was symptomatically indistinguishable from sporadic PD (Langston *et al*.,1983). MPTP was discovered to be a contaminant in the synthetic heroin that poisoned the drug users, and after it was recognized to function as a DA neurotoxicant, animal models were quickly developed, initially through repeated injections of MPTP in monkeys, providing the first effective non-human primate model of parkinsonism (Burns *et al*, 1983), and paving the way for the widespread use of MPTP as an *in vivo* neurotoxin. This molecule has been shown to be toxic in a large range of species and it is the tool of choice for investigations into the mechanisms involved in the death of dopaminergic neurons in PD, causing damage primarily to the nigrostriatal DA pathway with a profound loss of DA in the striatum and SN (Dauer and Przedborski, 2003). MPTP neurotoxicity arises from the formation of the toxic MPP^+ metabolite, which decreases ATP production, inhibits mitochondrial enzymes and increases ROS production (Figure 1) (Nicklas *et al*, 1985). Several studies have demonstrated that JNK, a protein that belongs to a family of stress kinases subject to transient activation in response to ROS, heat or osmotic shock and growth factors or inflammatory cytokines (Davis, 2000), is a key mediator of MPTP/MPP^+-induced neuronal apoptosis in animal models of PD (Nishi, 1997; Saporito *et al*., 2000). It has been shown that the SN of MPTP-treated mice presents increased levels of JNK and c-Jun (Silva *et al.*, 2005; Castro-Caldas *et al*., 2012), supporting the idea that this kinase is somehow involved in the MPTP-induced degeneration of dopaminergic neurons. Also, it was shown that JNK null mice are resistant to MPTP neurotoxicity (Hunot *et al*, 2004) and selective inhibitors of JNK protect against the neurodegeneration in the nigrostriatal dopaminergic neurons caused by MPTP (Saporito *et* al., 1999; Wang *et al.*, 2004). JNK activation is mediated through a sequential kinase cascade that results in the dual phosphorylation of the Thr-Pro-Tyr motif located in its activation loop (Davis, 2000), after which

JNK phosphorylates c-Jun, increasing its transcriptional activity and leading to the up-regulation of a number of genes involved in the control of cell survival and apoptosis (Kuan and Burke, 2005).

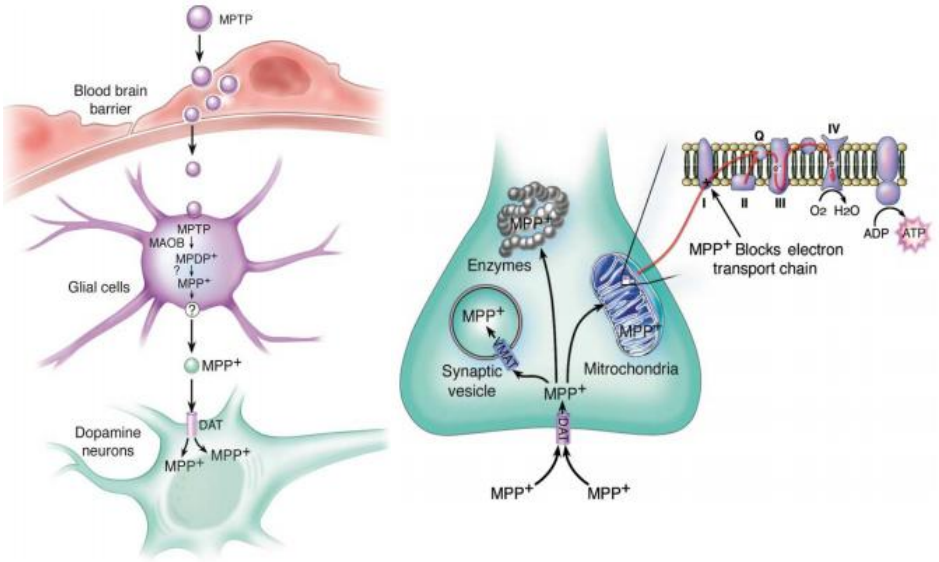

Figure 1 – Schematic representation of MPTP metabolism and intracellular pathways affected by MPP^+. Following systemic injection, MPTP readily crosses the BBB and is metabolized through the action of MAO-B in glial cells, converting MPTP to $MPDP^+$, which is then rapidly deprotonated (likely due to spontaneous oxidation) to the active neurotoxin MPP^+ (Przedborski *et al*, 2004). Afterwards, MPP^+ is released into the extracellular space where it enters dopaminergic neurons through selective uptake by the DA transporter. Once inside the neuron, MPP^+ enters the mitochondria through a mechanism dependent of mitochondrial transmembrane potential, where it binds to complex I, uncoupling the oxidation of NADH-linked substrates and consequently disrupting the flow of electrons along the electron transport chain. In Dauer and Przedborski (2003).

Among the toxic animal models of PD, rotenone represents one of the most recently used approaches (Betarbet *et al*., 2000). This substance is widely used around the world as an insecticide and pesticide, and it is a member of the rotenoids, a family of natural cytotoxic compounds extracted from various parts of Leguminosae plants (Hisata, 2002). Similarly to MPTP, rotenone is highly lipophilic and thus readily gains access to all organs including the brain. Furthermore, this neurotoxin also freely crosses all cellular membranes and can accumulate in subcellular organelles such as the mitochondria. After a single intravenous injection, rotenone reaches maximal concentration in the central nervous system within 15 minutes and decays to about half of this level in less

than 2 hours (Talpade *et al*., 2000). Once in the CNS, its brain distribution is heterogeneous, paralleling regional differences in oxidative metabolism.

Rotenone impairs oxidative phosphorylation by inhibiting NADH-ubiquinone reductase activity through its binding to the PSST subunit of the multipolypeptide enzyme complex I of the electron transport chain (Schuler and Casida, 2001). Beside its effects on mitochondrial respiration, rotenone also inhibits the formation of tubulin microtubules (Brinkley *et al*., 1974; Marshall and Himes, 1978), an effect relevant to the mechanism of dopaminergic neurodegeneration, as excess of tubulin monomers may be toxic to cells (Weinstein and Solomon, 1990). Rotenone has been used extensively as a prototypic mitochondrial poison in cell cultures, but less frequently in living animals. Behaviorally, rotenone-infused rats exhibit reduced mobility, flexed posture and in some cases rigidity and even catalepsy (Sherer *et al*., 2003).

The potent herbicide N,N'-dimethyl-4-4-4-bypiridinium, commonly known as paraquat, is another prototypic toxin known to exert deleterious effects through oxidative stress. Paraquat exhibits a striking structural similarity to MPP^+ and its toxicity is mediated by redox cycling with cellular enzymes that present nicotinamide adenine dinucleotide phosphate-diaphorase activity, such as neuronal form of nitric oxide synthase, yielding ROS (Przedborski and Ischiropoulos, 2005). Although it poorly crosses the BBB spontaneously (Shimizu *et al*., 2001), significant damage to the brain is seen in individuals who died from paraquat intoxication and epidemiological studies have suggested an increased risk for PD due to paraquat exposure (Liou *et al*., 1997), but at this time, the data and clinical evidence are still inconclusive (Berry *et al*., 2010). Some investigators have reported reduced motor activity and dose-dependent loss of striatal dopaminergic nerve fibers and SN neuronal cell bodies in paraquat treated mice (Bové *et al*., 2005). However, this toxin model has shown some contradictory results, variable cell death and loss of striatal DA content (Miller, 2007) and the effect of this compound in the nigrostriatal dopaminergic system are somewhat ambiguous (Freira and Koifman, 2012).

1.4.2. Genetic models of PD

With the identification of PD-associated genes and given the fact that the chemical-induced animal models do not show all the classical phenotypes of PD, animal models based on PD patient gene mutations have been created as well. Currently there are many genetic animal models of PD due in different model organisms, including mice, *Drosophila melanogaster* and *Caernorhabditis elegans*. There are also a variety of cell models that have been used to glean insight into how mutations in the various PD-related genes lead to neuronal dysfunction. These genetic models may better simulate the mechanisms underlying the genetic forms of PD, even though their pathological and behavioral phenotypes are often quite different from the human condition. A number of cellular and molecular dysfunctions have been shown to result from these gene defects (Blesa and Przedborksi, 2014) and some studies have reported alterations in motor function and behavior in mice models (Hinkle *et al*., 2012; Hennis *et al*., 2013), as well as sensitivities to complex I toxins like MPTP that differ from those of wild type mice (Nieto *et al*., 2012; Dauer *et al*., 2002). However, studies evaluating the integrity of the nigrostriatal dopaminergic system in these genetic models failed to find significant loss of dopaminergic neurons (Goldberg *et al*., 2003; Hinkle *et al*., 2012), suggesting that the recapitulation of the genetic alterations in mice is insufficient to reproduce the final neuropathological feature of PD.

Mutations in the *SNCA* gene are known to cause a rare form of autosomal dominant PD, either through isolated point mutation or gene multiplication (Lee and Trojanowski, 2006). Numerous α-synuclein transgenic mice have been reported using a variety of promoters. It has been shown that these mice models may have motor dysfunction and filamentous inclusions that initiate neurodegeneration (Giasson *et al*., 2002), although none of them show robust and progressive nigrostriatal degeneration (Ted *et al*., 2010). The phenotypic outcome of the overexpression of α-synuclein in mice depends heavily on the promoter used. Of the many vertebrate models, only the mouse

prion promoter A53T α-synuclein transgenic mice exhibit the full range of α-synuclein pathology that is observed in humans, including progressive age-dependent neurodegeneration (Chesselet *et al*., 2008). Other transgenic models also exhibit gradations of α-synuclein aggregation, but they lack the characteristic α-synuclein fibrils that are present in humans with PD and related α-synucleinopathies (Chesselet, 2008; Dawson et al, 2010).

The most frequent genetic cause of autosomal dominant PD is mutations in *LRKK2* (Zimprich *et al*., 2004). Several animal and cellular models of *LRKK2* have been reported and they have provided important insight into how mutations in this gene may lead to neurodegeneration in PD. Mutations in *LRKK2* that segregate with PD are concentrated in the GTPase and kinase domains but LRKK2 binding partners and regulators of kinase and GTPase activity have yet to be confirmed or clarified (Biskup and West, 2009). The majority of cases of *LRKK2* related PD is characterized pathologically by the presence of α-synuclein inclusions, suggesting that these two proteins share common pathogenic mechanisms (Ross *et al*., 2006). In cellular models, overexpression of disease causing mutations of *LRKK2* are toxic and toxicity is kinase and GTP-binding dependent (Smith *et al.*, 2006; West *et al*., 2007). Knockout of *LRRK2* in mice suggest that LRRK2 does not play a role in the development or maintenance of dopaminergic neurons (Andres-Mateos *et al*., 2009) and current transgenic mouse models are not very robust PD models. Most of these models have abnormalities in the nigrostriatal system such as stimulated DA neurotransmission or behavioral deficits, which are DA responsive (Dawson *et al*., 2010) but it is not yet clear why *LRRK2* transgenic models do not exhibit more substantial pathology. The utility of the current models is likely to be focused on how mutations in *LRRK2* lead to early dysfunction of the nigrostriatal dopaminergic system.

Regarding parkin, mutations in this gene are the most common genetic cause of early-onset PD (Marder *et al*., 2010). Knockout of this gene in *Drosophila* leads to mutant flies with reduced lifespan, male sterility and severe defects in both flight and climbing abilities, with decreased tyrosine hydroxylase levels observed in aged flies and specific degeneration of a subset of

dopaminergic neurons (Greene *et al*., 2003). However, none of the parkin KO mice have any substantial dopaminergic or behavioral abnormalities (Goldberg *et al*., 2005) and it has been shown that LB formation and neurodegeneration are parkin independent in a mouse model of α-synucleinopathy (von Coelln *et al*., 2006). Some of the parkin knockout mice have subtle abnormalities in the dopaminergic nigrostriatal circuit or the locus coeruleus noradrenergic system (von Coelln *et al*., 2004). Interestingly, overexpression of mutant human parkin in both *Drosophila* and mice lead to a progressive degeneration of dopaminergic neurons (Lu *et al*., 2009; Sang *et al*., 2007).

2. The Endoplasmic Reticulum and Oxidative Stress

It has been proposed that mitochondrial dysfunction and oxidative damage may play a critical role in both aging and neurodegenerative diseases (Beal, 2005). Mitochondrial oxidative phosphorylation is the primary source of high energy compounds in the cell and the dysfunction of mitochondrial metabolism leads to reduced ATP production, impaired calcium buffering and generation of ROS (Surmeier *et al*., 2011). In regards to PD, accumulating evidence indicates that oxidative stress contributes to the cascade of events leading to the degeneration of dopaminergic neurons in the SN. *Postmortem* brain analyses show increased levels of 4-hydroxyl-2-nonenal, a by-product of lipid peroxidation, carbonyl modifications of soluble proteins and DNA and RNA oxidation products (Dias *et al.,* 2013). Also, the link between oxidative stress and dopaminergic neurons is further supported by modeling the motor aspects of PD in animals with toxins that cause oxidative stress such as mitochondrial inhibitors like MPTP, rotenone or paraquat. This production of ROS may be the trigger for a dysfunction in the protein metabolism in the ER seen in sporadic PD. The ER serves many general functions, including protein processing, folding and the transport of membrane and secretory proteins, as well as the degradation of misfolded protein aggregates, providing and maintaining an exclusive oxidized environment of millimolar concentrations of Ca^{2+} to facilitate disulfide bond formation (Calì *et* al., 2011). This process is believed to

contribute to 25% of ROS generated by the cell (Tu and Weissman, 2004). Chronic ER stress through endogenous or exogenous insults may further increase oxidative stress via protein overload, impaired redox homeostasis, and calcium released from the ER, which in turn can augment the production and accumulation of mitochondrial ROS, thereby also influencing vital mitochondrial functions (Malhotra and Kaufman, 2011).

2.1 Endoplasmic Reticulum Stress

The ER is involved in several metabolic processes such as gluconeogenesis and lipid synthesis, while also being the major intracellular calcium reservoir in the cell (Chaudhari *et al*., 2014). Newly synthesized membrane and secreted proteins enter the ER in an unfolded state, where the protein maturation steps required for a proper folding occur. Folding might involve post-translational modifications, such as glycosylation or disulfide bond formation, assisted by a vast number of chaperones and modifying enzymes that also contribute to membrane integration (Braakman and Hebert, 2013). The protein-folding machinery in the ER is particularly challenged in specialized secretory cells (such as pancreatic β-cells), given the fact that these cells have a high demand for protein synthesis, constituting a constant source of stress.

Despite all the biological mechanisms dedicated to protein folding, a significant portion of newly synthesized polypeptides entering the ER fails to acquire a native conformation (Ruggiano *et al*., 2014). These misfolded molecules are retained in the ER and eventually become substrates of the ERAD, a set of quality-control mechanisms that clears the ER from harmful misfolded proteins (Brodsky, 2012) and plays a key role in ER homeostasis. Genetic ablation of several ERAD components leads to embryonic lethality in mice (Yagishita *et al*., 2005; Francisco *et al*., 2010) and the inactivation of the ERAD has tremendous consequences, resulting in the build-up of misfolded proteins in the lumen and membrane of the ER, a phenomenon also known as ER stress. This occurs when the folding capacity of ER exceeds the capacity of ER lumen to facilitate the disposal of misfolded proteins. Consequently, the ER

elicits a protective or adaptive response which involves an intricate set of signaling pathways that will be activated to compensate damage and to restore the cell back to its normal state of homeostasis (Walter and Ron, 2011). These biological processes are collectively called the UPR.

2.2 The UPR

The UPR is a global stress network that integrates information about the protein-folding status in the ER lumen to the nucleus and cytosol to decrease the unfolded protein load, controlling decisions on cell fate through a variety of complementary mechanisms (Schroder and Kaufman, 2005) (Fig. 2). The primary function of the UPR signaling is promoting the cell survival under hostile conditions. When cells undergo irreversible ER stress, UPR is responsible for the elimination of damaged cells through apoptotic mechanisms, some of which appear to be specific to ER stress and others that are included in general apoptotic pathways (Xu *et al*., 2005). Even though the UPR is linked to protein-folding stress under normal and pathological conditions, recently it has been shown that some of its components can regulate various processes, from lipid and cholesterol metabolism, to inflammation and cell differentiation (Rutkowski and Hedge, 2010). These alternative UPR outputs are thought to derive from the complex crosstalk between different stress and metabolic pathways, showing that UPR components are part of distinct regulatory modules that orchestrate the fine-tuning of essential homeostatic processes (Hetz, 2012).

Two distinct phases of cellular responses are observed in vertebrate cells undergoing ER stress. First, an inhibition of general protein translation occurs coupled with a selective degradation of mRNA encoding for certain proteins located in the ER and a bulk degradation pathway termed ER-phagy, a process that eliminates damaged ER and abnormal protein aggregates through the lysosomal pathway (Hetz, 2012). This first wave of cellular responses reduces the protein influx into the ER to allow adaptive and repair mechanisms that reestablish homeostasis. A second wave of events triggers a massive gene-expression response through the regulation of three distinct ER proximal UPR

transmembrane proteins: IRE1α, ATF6α and PERK. Under normal physiological conditions, all three effectors are negatively regulated by the ER chaperone GRP78/BIP, which suppresses their activity by binding to their luminal ends (Bertolotti *et al*., 2000). Under conditions of ER stress and increase in unfolded proteins, GRP78 dissociates from the transmembrane proteins, releasing the inhibition and eliciting the response. Activation of the ER pathways helps to fight the cellular stress through the combined actions of suppressing the translation of new proteins, inducing ER chaperones that promote protein refolding and activating the proteasome to degrade misfolded/unfolded proteins.

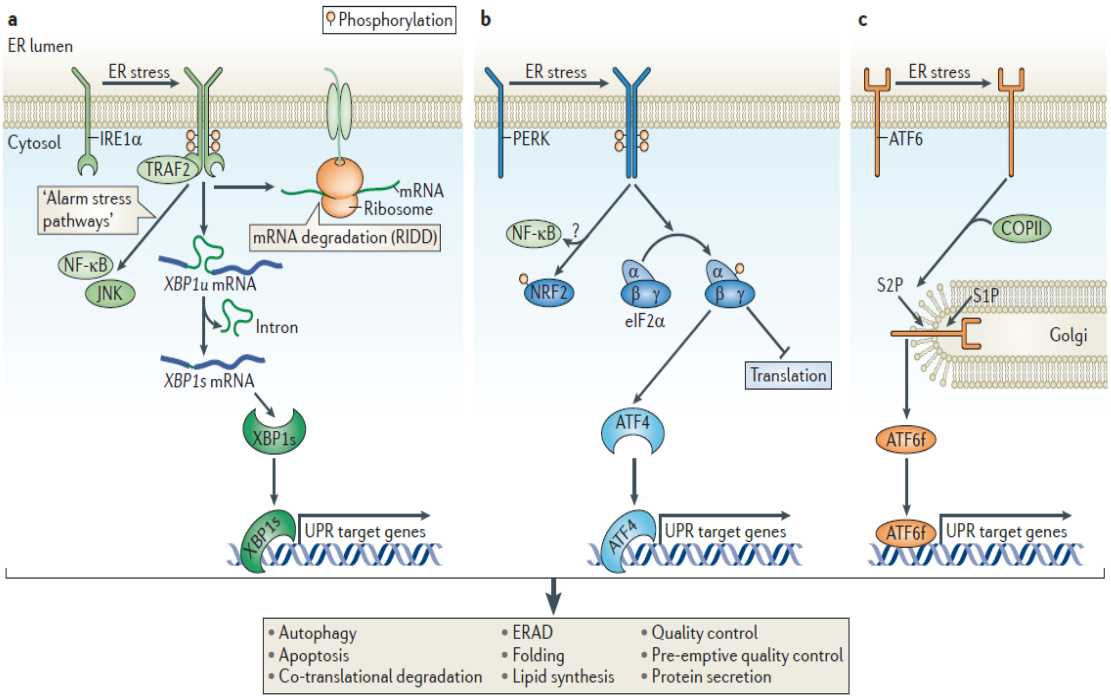

Figure 2 – Schematic representation of the UPR. The UPR stress sensors IRE1α, PERK and ATF6α transduce information about the folding status of the ER to the cytosol and nucleus to restore protein-folding capacity. Through its actions, the stress sensors mediate the attenuation of protein translation, the expression of protein-folding chaperones and many other genes involved in the UPR. In Hetz (2012).

2.2.1 PERK signaling

PERK is a type I ER transmembrane protein kinase with a luminal domain and a cytoplasmic domain that has kinase activity. Upon ER stress, BIP releases the luminal domain of PERK, which then dimerizes and autophosphorylates to become active. Following *trans*-autophosphorylation, this kinase phosphorylates the alpha subunit of eIF2, inactivating it by Ser-51 phosphorylation and attenuating protein translation. This inhibitory effect of translation helps to alleviate ER stress by decreasing the overload of misfolded proteins and thereby protecting the cells under conditions where proteins cannot achieve proper folding (Fels and Koumenis, 2006). Translation attenuation is followed by increased clearance of the accumulated proteins from the ER by ERAD and expression of pro-survival genes (Chakrabarti *et al*., 2012). Interestingly, eIF2α phosphorylation increases the translation of selective mRNAs that contain inhibitory upstream open reading frames within their 5'UTR that prevent translation in unstressed cells, such as the mRNA of ATF4 (Vattem and Wek, 2004). ATF4 is a member of the cAMP response-element-binding family of transcription factors and up-regulates a subset of UPR genes that function in resistance and control of oxidative stress, metabolism, protein folding and glutathione biosynthesis (Harding *et al*., 2000). Under severe ER stress conditions, ATF4 contributes to the induction of cell death through the control of the transcription of pro-apoptotic BCL-2 family members including p53 upregulated modulator of apoptosis and BIM, in addition to GADD34 and CHOP, a protein composed of an N-terminal transcriptional activation domain and a C-terminal bZIP that promotes apoptosis by the repression of Bcl2 expression and sensitization of cells to ER-stress inducing agents (Galehdar *et al*., 2010).

Furthermore, PERK can also phosphorylate Nrf2 (Cullinan *et al*., 2003), a member of a subfamily of bZIP transcription factors (Moi *et al*., 1994) that binds to a sequence known as the ARE, a response element found in many cytoprotective genes (Gao *et al*., 2014). In a stress-free cellular environment, Nrf2 is rapidly degraded by the proteasome. Degradation of Nrf2 is triggered by polyubiquitination through the cytoskeletal anchor Keap1/Cul3 ubiquitin ligase

that acts as a substrate adaptor to bring Nrf2 into the E3 complex (Kobayashi *et al*., 2004). In the presence of cellular stress, Nrf2 dissociates from Keap1 through the modification of reactive cysteine residues of Keap1 (Kobayashi *et* al., 2006) and is translocated to the nucleus, where it induces the expression of a wide variety of downstream target genes, that include several cytoprotective phase II detoxification and antioxidant enzymes and signaling proteins to regulate drug metabolism, antioxidant defense and oxidant signaling (Cullinan and Diehl, 2004). Through its regulation of oxidant levels and oxidant signaling, Nrf2 participates in the control of several functions such as autophagy, inflammasome signaling, apoptosis and UPR, exhibiting multiple protective effects against toxicity (Ma, 2013).

2.2.2. IRE1α signaling

IRE1α is a type I transmembrane protein ubiquitously expressed (Tirasophon *et al*., 1998) with dual enzymatic activities, consisting of an N-terminal ER luminal domain and a serine/threonine kinase domain plus a C-terminal RNase domain located in the cytosol (Lee *et al.,* 2008). Upon accumulation of unfolded/misfolded proteins in the ER and under conditions identical to those that activate PERK (Hetz *et al*., 2011), IRE1α dimerizes and oligomerizes while stimulating *trans*-autophosphorylation which leads to the activation of the RNase domain (Korennykh *et al*., 2009). Afterwards, activated IRE1α excises a 26-nucleotide intron from the XBP1 mRNA through its RNase domain, causing a translational shift in the codon reading frame that generates a new COOH terminal end and leads to the expression of a more stable and active transcription factor, XBP1s, that will translocate to the nucleus and regulate a subset of UPR target genes related to folding, ER/Golgi biogenesis and ERAD (Jiang *et al*., 2015), which differ in different tissues or under different conditions of ER stress (Acosta-Alvear *et al*., 2007). Furthermore, IRE1α can also degrade ER-bound mRNAs through cleavage at stem-loop and non-stem-loop sites, a process known as RIDD. RIDD helps to reduce the number of

proteins that enter the ER, further alleviating ER stress (Hollien *et al*., 2009). In the event of persistent ER stress, activated IRE1α can also promote apoptotic pathways by activating ASK1 and JNK, through interaction with TRAF2 (Urano *et al*., 2000; Nishitoh *et al*., 2002).

2.2.3. ATF6α signaling

Another UPR pathway is mediated by ATF6α, a type II ER transmembrane protein that contains bZIP domains in its cytosolic region (Haze *et al*., 1999). ATF6α is synthesized as an inactive precursor, bound to the ER by a transmembrane segment. Under ER stress conditions, ATF6α precursor translocates to the Golgi where it is cleaved in a process termed regulated intramembrane proteolysis. Firstly, ATF6α is cleaved by a site 1 protease which removes most of its luminal domain, followed by the removal of an intramembrane region by a site 2 protease, releasing an active N-terminal 50 kDa cytosolic fragment (ATF6f) (Nakka *et al*., 2014). This fragment operates as a transcriptional factor by binding to the ER stress response element within the promoter region of target genes, which will then up-regulate the expression of many UPR genes related to ERAD (Shen and Prywes, 2005).

Both processing of ATF6 and IRE1α-mediated splicing of XBP1 mRNA are required for full activation of the UPR. Furthermore, it is thought that the ATF6 and IRE1α pathway merge through the regulation of XBP1 activity: ATF6 increases the amount of *XBP1* mRNA whereas IRE1α removes the 26-nucleotide intron, increasing XBP1 transactivation potential. Together with XBP1s, ATF6f will increase the transcription of target genes that expand ER size and increase its protein folding capacity to promote cell survival (Lee *et al*., 2002).

2.3 ER Stress in PD

Some reports have revealed that the UPR is an early event in PD and the presence of ER stress in human tissue derived from PD patients has been reported (Hoozemans *et al*., 2012). Immunoreactivity for phosphorylated PERK

and eIF2α in dopaminergic neurons of the SN has been described in PD *post-mortem* tissue, and the neurons presenting activated PERK were also positive for α-synuclein inclusions (Hoozemans *et al*., 2007). Furthermore, it has been demonstrated that ER stress-responsive proteins such as homocysteine-induced endoplasmic reticulum protein are upregulated in the SN of PD patients and co-localize with LBs (Conn *et al*., 2004; Slodzinski *et al*., 2009). Cellular studies in yeast have shown that overexpression of wild type and mutant α-synuclein triggers chronic ER stress inducing cell death (Cooper *et al*., 2006) and reports in complementary model organisms demonstrated that the earliest defect following α-synuclein expression is a block in ER to Golgi vesicular trafficking (Smith *et al*., 2005; Gitler *et al*., 2008). The involvement of the UPR in PD has also been shown in cellular and *in vivo* models using 6-OHDA, MPTP and rotenone (Blesa and Przedbroski, 2014). Generation of ROS by these neurotoxins leads to a rapid accumulation of oxidized proteins that can activate the UPR. Moreover, neuronal cells treated with PD-triggering toxins present phosphorylation of IRE1α and PERK as well as induction of their downstream targets (Ryu *et al*., 2002). Experiments carried out using neuronal cultures from PERK KO mice revealed an increased sensitivity of these cells against treatment with 6-OHDA, suggesting that neurons lacking this protein were unable to properly activate the UPR and that an early UPR response may be neuroprotective for the dopaminergic neurons (Lindholm *et al*., 2006). Similarly, ATF6α deficient mice are more susceptible to neurotoxin-induced neurodegeneration at the SN (Egawa *et al*., 2010)

Further evidence for a role of the UPR in PD pathogenesis comes from a juvenile onset autosomal recessive form of PD that is caused by mutation of the parkin gene, which compromises the ubiquitin ligase function of the protein. This leads to the accumulation of cytotoxic fibrils and protein aggregates in the ER of SN neurons (Imai *et al*., 2001), that result in ER stress and consequently cell death. Expression of wt parkin, on the other hand, can restore proteasome function, which was shown to be impaired in SN neurons in PD (McNaught and Jenner, 2001), and it has a pro-survival activity against ER stress due to modulation of ERAD/proteasome pathway (Imai and Takashi 2004).

Although these reports suggest that ER stress occurs in affected SN neurons in PD brain and that it may trigger ROS production and redox deviation in the ER, general characterization of ER stress markers is still very poor and proximal signaling components (ATF6α, XBP1s, etc.) remain to be properly measured. The precise mechanisms of interplay between oxidative stress and ER stress in dopaminergic neurons have been sparsely described and whether ER stress plays a role in oxidative stress and anti-oxidant elicited neuronal response is currently unknown, as the mechanisms leading to ER stress in PD and the actual impact of the UPR on the degeneration cascade in the disease are just starting to be uncovered (Mercado et al. 2013).

3. Glutathione S-transferases

GSTs are a class of abundant proteins found in most tissues that function as xenobiotic metabolizing enzymes in eukaryotes. This supergene family of enzymes catalyses the conjugation of nucleophilic thiol-reduced GSH to a variety of electrophiles, forming water-soluble GSH conjugates that are readily transported out of the cell via membrane-bound efflux pumps (Board and Menon, 2013). They are an important cellular defense against numerous artificial and naturally occurring environmental agents (Strange *et al*., 2000) and GSTs' substrates include polycyclic aromatic hydrocarbon epoxides derived from the catalytic actions of phase 1 cytochrome P450s as well as numerous by-products of oxidative stress (Strange *et al*., 2000). In addition to their role in detoxification, GST isozymes have other characteristics and functions such as the regulation of MAPKs and participation in steroid synthesis, tyrosine degradation and dehydroascorbate reduction (Tew and Townsend, 2012; Wu and Dong, 2012). Furthermore, the deletion of certain genes that encode for GST isoforms has been linked to an increased susceptibility to bladder, colon, skin and possibly lung cancer (Hayes and Pulford, 1995) and linked to a change in drug response (Layton *et al*., 1999; Roy *et al*., 2001).

GSTs are divided into two main families: cytosolic or soluble GSTs, and microsomal. Microsomal GSTs have been described as membrane-associated proteins and are structurally distinct from cytosolic GSTs, though they still

maintain an ability to catalyze the conjugation of GSH to electrophiles (Hayes *et al*., 2005). Cytosolic GSTs are expressed in all aerobic organisms (Ketterer, 2001) and a single GST unit consists of an N-terminal α/β-domain that operates as a GSH binding site and an all-α.helical domain for substrate binding that facilitates catalysis through proton abstraction from GSH in proximity to the GSH binding site (Dirr *et al*., 1994). Structural studies have shown that GSTs are typically monomeric but catalytically active as homo- or heterodimers composed of 25-30 kDa subunits (Hayes *et al*., 2005) and are divided into eight classes: alpha, kappa, mu, pi, sigma, theta, zeta and omega (Mannervik *et al*., 2005).

In the human brain, the active GSTs are composed of dimers containing alpha, mu or pi class GST monomers. GST mu is the most highly expressed isoform in the brain followed by GSTP and then GST alpha (Smeyne *et al*., 2013). Cellular localization studies have shown that GSTP and GST mu are expressed in neurons, astrocytes and oligodendrocytes, but it has been found that only GST pi is found in the dopaminergic neurons of the SN (Smeyne *et al*., 2007), a region that is particularly sensitive to oxidative stress due to the presence of endogenous dopamine, iron and neuromelanine. The distribution of GSTs in the brain appears to also be age dependent (Carder *et al*., 1990).

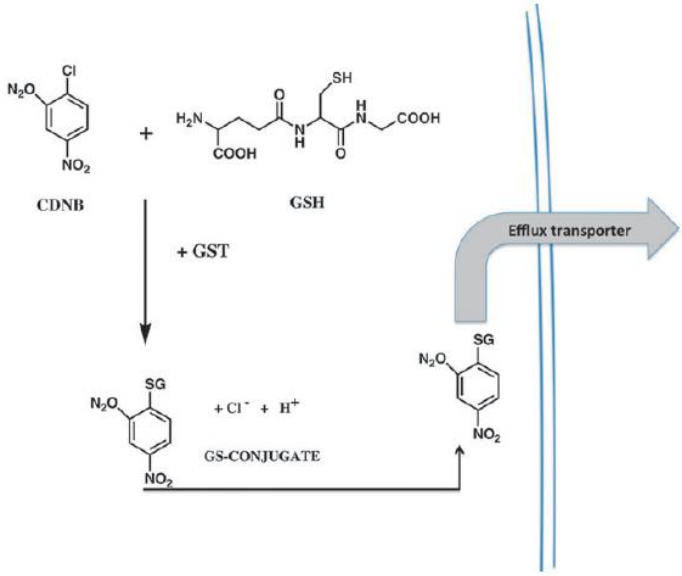

Figure 3 – Detoxification scheme for glutathione conjugation. Scheme of detoxification of 1-chloro-2, 4-dinitrobenze through catalytic thioether formation. GST – Glutathione S-Transferase; GSH – Glutathione. Tew and Townsend, 2012 (adapted).

3.1. Glutathione S-transferase Pi

GSTP is one of the most extensively studied GSTs and has been implicated in the protection of cells from ROS-inducing agents due to its ability to alter levels of cellular glutathione in response to production of ROS, ameliorating the oxidative milieu (Tew and Ronai, 1999). Mainly found in the cytoplasm and widely distributed in a range of tissues (Suzuki *et al*., 1987), GSTP is induced by exposure to electrophiles. At the transcriptional level GSTP is regulated mainly by the GSTP enhancer 1 element which is recognized by Nrf2 (Suzuki *et al*., 2005). In contrast to the mouse that has two pi class GST genes (Bammler *et al.,* 1994), humans have a single functional GSTP gene termed *GSTP1* located in chromosome 11q13 (Board *et al*., 1989). Single nucleotide polymorphisms (SNPs) in the *GSTP1* gene have been found and correlated with several malignancies such, as the development and progression of Hodgkin's and non-Hodgkin's lymphoma, and cancer drug response (Lourenço *et al*., 2009). These polymorphisms cause a steric change at the substrate binding site of the enzyme without affecting the GSH binding affinity (Ali-Osman *et al*., 1997).

GSTP has also been shown to protect cells from ROS by modulating S-glutathionylation of proteins following oxidative and nitrosative stress, a post-translational modification that consists of the addition of GSH to low pKa cysteine residues of target proteins (Tew 2007). Protein cysteines are evolutionarily highly conserved and sparingly used throughout the proteome, suggesting a tight control of cysteine-dependent biological functions. They have a wide-ranging reactivity towards oxidants, which depends on their pKa (Dalle-Donne *et al*., 2007; Rutkevich and Williams, 2012). It is currently believed that the type of cysteine oxidation controls the biological response as well as the fate of the oxidized protein (Finkel, 2011). Certain types of cysteine oxidation may occur preferentially in subcellular compartments where the redox environment may be facilitating these oxidations, such as the ER (Chakravarthi *et al*., 2006). S-glutathionylation generally occurs when a cysteine within the protein forms a disulfide bond with GS^-, which can occur either in response to

endogenous oxidative or nitrosative stress mediated signaling events or from exposure to external environment drug treatments (Townsend, 2007). A wide range of chemicals can induce S-glutathionylation such as hydrogen peroxide, glutathione disulfide, diamide and various nitric oxid donors (Townsend, 2007; Townsend *et al*., 2008). S-glutathionylation has been associated with the stabilization and protection of proteins against irreversible oxidation of critical cysteine residues, and with the regulation of protein functions and transcription. These proteins include enzymes with catalytically important cysteines, particularly those involved in protein folding and stability, nitric oxide regulation and redox homeostasis, such as kinases, phosphatases, heat shock proteins, proteins involved in energy metabolism and transcription factors (Tew *et al*., 2012).

It has been shown that Keap1 is modified by S-glutathionylation (Zhang et al 2010). This protein has high cysteine content, making it an excellent candidate for this post-translational modification. Interestingly, GSTP itself is subject to S-glutathionylation, which reduces its enzymatic activity against chemical substrates and promotes its multimerization (Dalle-Donne et al 2007). Altered levels of S-glutathionylation in some proteins have been associated with numerous pathologies, many of which linked to stress within the ER (Janssen-Heininger et al 2013).

Besides being involved in cell redox homeostasis, GSTP also has a number of other functions such as catalyzing degradation of nitro-compounds (Lo Bello *et al*., 2001), and participating in reactions involving stress kinases (Adler *et al.,* 1999). In regards to the latter, it has been shown that GSTP1 monomer can act as a ligand-binding protein controlling the catalytic activity of JNK (Adler *et al.,* 1999; Castro-Caldas *et al*., 2012), an interaction that involves the C-terminal region of GSTP and of JNK (Monaco *et al.,* 1999), preventing c-Jun phosphorylation and inhibiting the subsequent trigger of the cell death cascade. Under conditions of oxidative stress,
GSTP dissociates from JNK, which may then be phosphorylated and consequently phosphorylate its downstream substrates (Yin *et al*., 2000). It has also been shown that after systemic administration of MPTP, GSTP expression

is significantly increased in glial cells in the vicinity of dopaminergic neurons cell bodies and fibers (Castro-Caldas *et al*., 2009).

The creation of two GSTP KO strains has been reported, one involving mouse *GSTP1* (GSTP1-1) and the second inactivating both genes (Henderson *et al*., 1998), and all studies reported so far involved the double KO mouse strain (GSTP1/2 null mice). These mice do not present striking or lethal phenotypes and have been mainly used to explore the role of GSTP in tumourigenesis (Board, 2007). The results suggest that the contribution of GSTP to tumourigenesis is multifaceted; GSTP1/2 null mice are more sensitive to a two-stage skin tumourigenesis model (Henderson *et al*., 1998) showing a detoxification role for GSTP.

Furthermore, it has been shown that GSTP KO mice are more susceptible to the neurotoxic effects of MPTP than their wild type counterparts. Administration of MPTP induced a demise of nigral dopaminergic neurons together with the degeneration of striatal fibers at an earlier timepoint in the GSTP KO mice when compared to the wild type counterparts. It has also been shown that *in vivo* GSTP can act as an endogenous regulator of the MPTP-induced cellular stress by controlling JNK activity through protein-protein interactions (Castro-Caldas *et al*., 2012). The various contributions of GSTP to carcinogenesis and other biological processes reflect the mounting evidence that some GSTs participate in cell signaling pathways that are independent of their drug and xenobiotic detoxification roles.

3.1.1. GSTP in PD

There have been multiple reports concerning a role for GSTP in PD, both in cellular and animal models. It was demonstrated that over-expression of wild-type *GSTP1* before treatment with rotenone effectively protected Neuro2A cells by reducing oxidative stress, cell death, neurite loss and attenuating PERK activation and CHOP induction, all of which are important components of neurodegeneration in PD progression (Oakes and Papa, 2015; Shi *et al.,* 2009), and *GSTP1* mutants with low catalytic activity had a diminished effect, providing compelling evidence that GSTP's biological functions effectively reduced

oxidative stress and the associated ER stress (Shi *et al*., 2009). Also, using primary cultured dopaminergic cells harvested from the SN of MPTP resistant mice (Hamre *et al*., 1999), Smeyne and collaborators have shown that by inhibiting GSTP there was an increase in the amount of MPP^+-induced neuronal death (Smeyne *et al*., 2007). Administration of MPTP to mice lacking GSTP also showed altered protein ubiquitination and increased susceptibility to UPS damage and inactivation (Carvalho *et al*., 2012). Furthermore, reports have shown that by mutating the parkin gene in *Drosophila* and deleting the *GSTS1* gene, the loss of dopaminergic neurons is enhanced and overexpression of *GSTS1* ameliorates this neurodegeneration (Whitworth *et al*., 2005).

The analysis of the SN in the *post-mortem* brain of PD patients has revealed a substantial reduction of GSH levels and low activity of enzymes related to the *de novo* synthesis of GSH that seems to be specific to this pathology since changes in GSH were not detected in other neurodegenerative diseases (Di Monte *et al*., 1992; Sian *et al*., 1994; Pearce *et al*., 1997). Another study that compared ventricular cerebrospinal fluid from PD and normal control subjects, has shown differences in proteins expression in PD individuals, namely in *GSTP1-1* (Maarouf *et al*., 2012). Epidemiological studies have shown that decreased GSTP expression is a significant risk factor in PD and that GSTP wild type allele is an individual protective genetic trait in idiopathic PD (Golbe *et al*., 2007). Furthermore, it has been found an association between the A313G polymorphism in *GSTP1* and sporadic PD (Kelada *et al.,* 2003; Vilar *et al*., 2007), suggesting that the decreased conjugation of some GSTP substrates may be relevant to the etiology of PD.

4. TUDCA as a therapeutic approach

4.1 UDCA and TUDCA: endogenous functions and therapeutic properties

Bile acids are detergent molecules synthesized in the liver from neutral sterols (Russell and Setchell, 1992). In most animals (including humans) bile acids are produced mainly from the cholesterol metabolic pathway, and

complete synthesis requires several enzymes in processes tightly regulated by nuclear hormone receptors and other transcription factors (Chiang, 2004). Bile acids are the major constituents of the bile and play crucial biological functions such as the solubilization of dietary fats and fat-soluble vitamins to improve absorption in the intestinal lumen. There has been a growing interest in the last decades in these acidic steroids since the discovery of their role in important physiological phenomena, including liver and intestinal pathology and pharmacology (Paumgartner and Beuer, 2004). In fact, some bile acids are cytotoxic molecules involved in increased cell proliferation and cancer development in the intestinal tract (Bayerdorffer *et al*., 1993), and cell death by necrosis and apoptosis, a key event during hepatobiliary diseases (Patel and Gores, 1995). However, not all bile acids are toxic and it has been suggested that this is due to subtle changes in their chemical structure (Hofmann and Roda, 1984): hydrophobic bile acids can induce cell death in liver cells during cholestasis, by activating both ligand-dependent and –independent death receptor oligomerization and signaling the mitochondrial pathway of apoptosis (Faubion *et al*., 1999; Yerulshami *et al*., 2001), while hydrophilic bile acids can be cytoprotective through the activation of cell survival pathways such as MAPK and phosphoinositide 3-kinase, preventing mitochondrial dysfunction and consequently apoptosis (Schoemaker *et al*., 2004).

UDCA is an endogenous hydrophilic bile acid currently approved for the treatment of certain liver diseases such as primary biliary cirrhosis, due to its choleretic effects and ability to protect hepatocytes from hydrophobic bile acids (Lazaridis *et al*., 2001). UDCA accounts for 4% of the bile acid pool in the human body (Bachrach and Hofmann, 1982) and there is strong evidence that its cytoprotective effects result from the ability to reduce the apoptotic threshold in several cell types by modulating classical mitochondrial pathways (Rodrigues *et al*., 1998). This steroid can also activate specific nuclear receptors and G protein-coupled receptors influencing the expression of genes that encode proteins involved in the regulation of glucose, fatty acid, lipoprotein synthesis, energy metabolism and the regulation of their own synthesis (Hylemon *et al*., 2009). Further studies have shown that UDCA as well as its taurine conjugate TUDCA also inhibit oxygen-radical production and reduce caspase activation

(Amaral *et al*., 2009). Moreover, it has also been shown that UDCA and TUDCA can prevent UPR dysfunction and ameliorate ER stress. It does so by improving the protein folding and assisting in the transfer of mutant proteins and also through the inhibition of eIF2α (Omura *et al*., 2013). Other articles have shown that TUDCA can activate PERK (Gani *et al*., 2015; Liu *et al*., 2015). UDCA and TUDCA have also been shown to have ameliorating effects in inflammatory metabolic diseases including atherosclerosis, diabetes, renal disease and stroke (Vang *et al*., 2014).

4.2 TUDCA in neurodegenerative diseases

In recent years, neuroprotective functions have been attributed to TUDCA (Rodrigues and Steer, 2000). Studies *in vitro* using millimolar concentrations of TUDCA have demonstrated its inhibitory effects on the thermal aggregation of different proteins (Song *et al*., 2011; Berger and Haller, 2011). It has been thoroughly demonstrated that TUDCA can cross the BBB in humans (Parry *et al*., 2010), reducing the accumulation of toxic aggregates in different experimental models of neurodegenerative diseases, acting as a mitochondrial stabilizer and anti-apoptotic agent with cytoprotective properties (Keene *et al*., 2002; Elia *et al*., 2015). Furthermore, TUDCA is bioavailable and presents a low toxicity profile, which represents a therapeutic advantage and has led to an increasing attention as potential treatment for neurodegenerative conditions.

The protective role of TUDCA has been extended to numerous mouse models of neurological disorders. Several reports have shed light on the neuroprotective effects of TUDCA in Alzheimer's disease (Ramalho *et al*., 2008). In vitro studies have shown that TUDCA inhibits Aβ-induced apoptosis (Solá *et al*., 2006; Viana *et al*., 2010). Using primary rat cortical neurons, it was shown that TUDCA activates pro-survival signaling cascades decreasing Aβ mediated apoptosis (Solá *et al*., 2003). In regards to animal models, TUDCA treatment significantly attenuated Aβ deposition in the brain of APP/PS1 mice after disease onset while also reducing the amyloidogenic processing of

amyloid precursor protein, ameliorating memory deficits (Nunes *et al*., 2012; Lo *et al*., 2013). This is accompanied by a decrease in the glial activation and reduced pro-inflammatory cytokine expression, partially rescuing synaptic loss (Dionísio *et al*., 2015). Furthermore, it was shown that TUDCA modulates synaptic deficits induced by Aβ, preventing the reduction in dendritic spine number and decreasing spontaneous miniature excitatory synaptic activity (Ramalho *et al*., 2013).

Concerning HD, cell cultures treated with TUDCA significantly increased neuronal survival by inhibiting the release of cytochrome c in isolated mitochondria, DNA fragmentation and caspase activation, consequently inhibiting apoptosis (Keene *et al*., 2001; Rodrigues *et al*., 2000). The protective effect of TUDCA is also seen in toxin models of HD, with TUDCA preventing striatal degeneration and ameliorating locomotor and cognitive deficits *in vivo* (Keene *et al*., 2001) and genetic models of HD, in which systemic administration of TUDCA led to a significant reduction in striatal neuropathology (Keene *et al*., 2002). In fact, clinical trials are currently underway to study the tolerability and efficacy of TUDCA in patients with HD and amyotrophic lateral sclerosis (Cortez and Sim, 2014).

In the context of PD, a recent study that screened more than 2000 compounds identified UDCA as a highly promising drug therapy for future neuroprotective trials in PD (Mortiboys *et al*., 2013). TUDCA has also been found to play a role as a neuroprotective molecule in PD by protecting against apoptosis and regulating JNK activity and cellular redox thresholds. TUDCA treatment conferred protection against rotenone-induced toxicity and non-transgenic lines were fully protected by this bile acid, with some transgenic strains showing increased survival with TUDCA treatment *(*Ved *et al*., 2005). In mice models, the neuroprotective role of TUDCA has also been validated against MPTP toxicity. In a study with mice lacking GSTP, pre-treatment with TUDCA significantly reduced the depletion of dopaminergic neurons and dopaminergic fiber loss caused by MPTP. TUDCA also modified the cellular environment and attenuated the deleterious events of MPTP by blocking ROS production and JNK activation in GSTP null mice (Castro-Caldas *et al*., 2012).

Further support for the protective role of TUDCA in PD comes from reports of the transplantation of nigral dopamine neurons into rodent models: parkinsonian rats were transplanted with nigral dopamine neurons from fetal rats incubated with 50 μM TUDCA or saline. Rats treated with dopamine neurons incubated in TUDCA exhibited a significant reduction in cell death rates, as histological analysis of the transplanted cells revealed a significantly greater number of tyrosine-positive cells in the TUDCA-treated cells versus the saline-treated cells (Duan *et al*., 2002). These results suggest that TUDCA can enhance survival of transplanted dopamine neurons via reduction of apoptosis.

5. Objectives

Previous studies in our laboratory have shown that there may be a role for GSTP in PD. We hypothesized a potential neuroprotective role of this GST isoform, and intend to clarify its role by studying the Nrf2-mediated regulation and ER stress response in the context of MPTP-induced brain lesions. The work relied on the use of a GSTP KO mouse model and aimed to contribute to a better understanding of the molecular mechanisms underpinning loss of dopaminergic neurons in PD, which may lead to the development of novel therapeutic strategies.

The main objectives of this study were:

(a) Characterize the effects of the MPTP insult on the ER stress response in C57BL/6 wt and GSTP null mice brain;

(b) Determine the effect of GSTP deletion in the context of MPTP-induced ER stress;

(c) Evaluate the potential neuroprotective effect of TUDCA on MPTP oxidative driven ER stress.

II. MATERIALS and METHODS

1. Animals and Treatment

All animal experiments were carried out in accordance with the institutional procedures and Portuguese and European guidelines for the care and use of animals (*Diário da República, 2.ª série N.º 121* of 27 June 2011; and 2010/63/EU European Council Directive), and methods were approved by the Direcção Geral de Alimentação e Veterinária (DGAV, reference 021944 and the Ethical Committee for Animal Experimentation of the Faculty of Pharmacy, University of Lisbon.

C57BL/6 Gstp1/p2 null mice (Cancer Research UK) were re-derived and maintained at the Gulbenkian Institute of Science Animal House (Oeiras, Portugal). This line has a double-knockout of both Gstp genes (Gstp1 and Gstp2), deleted by homologous recombination (Henderson *et al*., 1998). The animals were housed under standardized conditions on a 12-h light–dark cycle with free access to a standard diet and water ad libitum.

Animals were treated only with TUDCA or MPTP, or treated with TUDCA prior to MPTP administration. TUDCA was dissolved in phosphate-buffered saline (PBS), pH 7.4, and was injected intra-peritoneally (i.p.) for three consecutive days (50 mg/kg body weight). MPTP was administered i.p. at a single dose of 40 mg/kg (Saporito et al. 2000). In TUDCA and MPTP co-treatments, TUDCA daily administration (50 mg/kg body weight) began on day 0, followed by i.p. administration of MPTP at a single dose (40 mg/kg body weight) on day 3, 6 h after the last TUDCA injection (Keene *et al*., 2001; Castro-Caldas *et al*., 2009). Control mice received saline alone. Mice were sacrificed 3 or 6 h after neurotoxin or vehicle administration. Samples from saline-treated and TUDCA-treated mice were collected prior to MPTP injection.

The time course studies were carried out in three independent experiments (n=3) with groups of three to six mice. The schematic chronogram of TUDCA and MPTP administration is shown in Figure 4.

After being anesthetized with sodium pentobarbital (50 mg/kg, i.p.), mice were decapitated and brains were quickly removed and placed in fresh PBS. Brains were then placed on their ventral surface onto a mouse brain matrix (Agar Scientific), and a slice between Bregma −2.5 and Bregma −3.8 was

isolated. This removed brain slab was placed flat and the entire cortex region was dissected. The specific pieces of interest were flash frozen under liquid nitrogen until further use. Preliminary studies have shown that in the saline control samples the evaluated parameters did not change through the time course; therefore, collection of control tissues was carried out at injection day 1, reducing the number of animals needed in these studies. The time course study was carried out in three independent experiments.

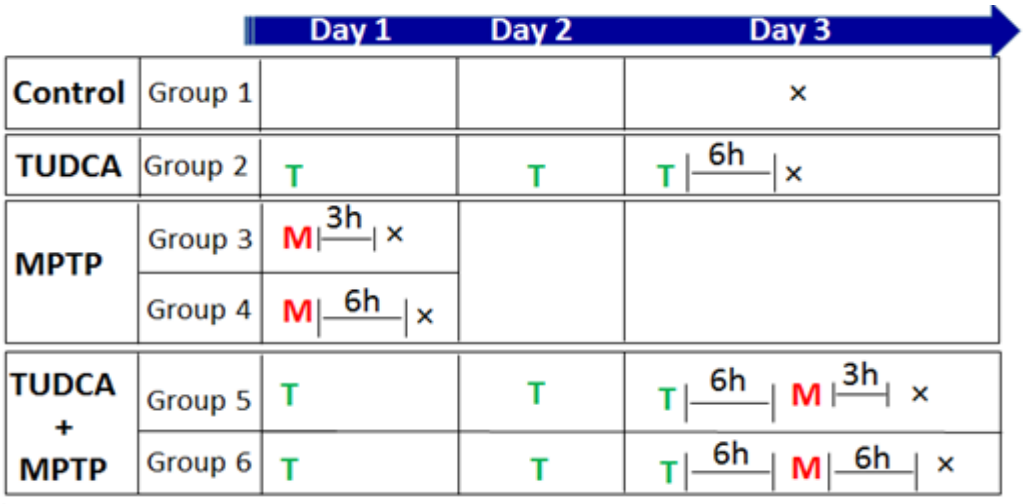

Figure 4 - Schematic representation of C57BL/6 wild type and GSTP null mice treatment course. Mice were i.p. injected with TUDCA (50 mg/kg body weight) for three consecutive days. MPTP was administered i.p. at a single dose of 40 mg/kg body weight.
Mice were divided into 4 main groups: i) control mice that received saline (group 1); ii) mice that received only TUDCA injections for 3 consecutive days, and were sacrificed 6 h after the last TUDCA administration (group 2); iii) mice treated with MPTP, that were sacrificed 3 h (MPTP, 3h) or 6 h (MPTP, 6h) after MPTP injection (group 3 and 4); iv) mice that received daily injection of TUDCA beginning on day 1, followed by i.p administration of MPTP on day 3, and were sacrificed 3 h (T+M, 3h) or 6 h (T+M, 6h) after MPTP injection (group 5 and 6). Samples for Western blotting were taken 6 h after the last TUDCA injection, or 3 and 6 h after MPTP administration.
(M – MPTP administration; T – TUDCA administration; X – Animal Sacrifice)

2. Western Blot Analysis

Tissue samples were homogenized in ice cold PBS using a Potter-Elvehjem homogenizer, followed by a 10 min centrifugation at 4ºC, after which the pellet was collected. For total protein extracts, lysis buffer ($NaVO_3$ 200 mM, NaF 1 mM, DTT 1 mM) plus Complete Mini protease inhibitors cocktail was added to the pellet. Lysates were sonicated on ice, five times for 5 s each, centrifuged at 13,000×*g* for 15 min at 4ºC, and the supernatant was recovered. Total Protein concentration was determined by the Bradford method (Bradford, 1976) using Bio-Rad's Protein Assay Reagent.

Tissue extracts with 100 µg of total protein were added (1:5) to denaturing buffer (0.25 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 40% glycerol, 0.2% bromophenol blue, 1% β-mercaptoethanol), boiled for 5 min, resolved on 12,5% SDS-polyacrylamide gel electrophoresis, and electrotransfered to PVDF membrane. The membrane was blocked with 5% non fat dry milk in Tris-buffered saline with 0.1%Tween-20, for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies (Table 2). After the membranes were washed for three times with Tris-buffered saline with 0.1% Tween-20 for 15 minutes each and incubated with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. Afterwards, the membranes were rinsed in Tris-buffered saline with 0.1% Tween-20 three times for 15 minutes each, and the immunocomplexes were detected with Pierce™ ECL Western Blotting Substrate (32106; ThermoScientific) or SuperSignal West Femto Maximum Sensitivity Substrate (34096; ThermoScientific). Densitometric analyses were performed using the Image Lab software Version 5.1 Beta after scanning with ChemiDoc™, both from Bio-Rad Laboratories (Hercules, CA, USA). Membranes were then stripped, with stripping solution (1.5% glycine, 40% glacial acetic acid, 1% SDS, 10% Tween 20) for 10 min, and rinsed several times in TBS-T. Stripped membranes were then blocked as previously described and incubated with mouse anti-β-actin primary antibody, followed by incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody. β-actin expression was used as a loading control.

Table 2 – List of primary antibodies used in the Western blot assays.

Primary Antibody	Molecular Weight	Secondary Antibody	Supplier	Reference
ATF6	70 kDa	Mouse	ThermoScientific	MA5-16172
β-Actin	42 kDa	Mouse	Sigma-Aldrich	A5441
CHOP	19 kDa	Rabbit	EnoGene	E11-0863B
eIF2α	36 kDa	Rabbit	Cell Signaling	9722
p-eIF2α	36 kDa	Rabbit	Cell Signaling	9271
IRE1α	110 kDa	Rabbit	Cell Signaling	3294
Nrf2	70 kDa	Mouse	R&D	MAB3925
PERK	130 kDa	Rabbit	Cell Signaling	3192
p-PERK(Thr980)	130 kDa	Rabbit	Cell Signaling	3179

3. Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with ice-cold PBS, followed by 4% paraformaldehyde–PBS, pH 7.4. After perfusion, brains were quickly removed and fixed by immersion, at 4°C for 24 h, in a solution containing 85 ml of 2% paraformaldehyde and 15 ml of saturated picric acid per 100 ml of fixative. After washing several times with PBS containing 15% sucrose and 0.1% sodium azide, brains were processed for cryostat sectioning. Cryostat coronal sections (20 µm thick, Bregma 0.38) were permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature and then pretreated with blocking solution (10% bovine serum in PBS), for 1 h at room temperature. Incubation with primary antibody anti-Nrf2 (ab31163; Abcam) was performed overnight at 4°C. After extensive rinsing in PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (Alexa Fluor 488 A11008; ThermoScientific) and Hoechst, for 1 h at room temperature. Finally, sections were washed with PBS, mounted in fluorescent mounting medium observed under an Axioskop microscope (Carl Zeiss) with an attached Leica DFC490 camera, and photographed using Image Manager 50 software (Leica Microsystems, Inc.).

4. Statistical Analysis

Data comparisons were conducted with one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Differences between wild type and GSTP KO groups were analyzed by two-way ANOVA with Bonferroni post hoc. Analysis and graphical presentation were performed using GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as mean ± standard error of the mean (SEM).

III. RESULTS

1. The expression levels of mediators of the UPR pathways are altered in GSTP KO mice

Even though recent studies show that ER stress may trigger ROS production and redox deviation in the ER, the precise mechanisms of interplay between oxidative stress and ER stress in the dopaminergic neurons have been sparsely described and whether ER stress plays a role in oxidative stress and anti-oxidant elicited neuronal response is currently unknown.

In this work we started by analyzing the protein expression levels of the three main mediators of the UPR, ATF6α, IRE1α and PERK in mice brain samples under MPTP-induced oxidative stress and/or treated with the chemical chaperone TUDCA. Moreover, we also wanted to look for differences between the expression of these UPR mediators in both wt and GSTP KO mice brain. In fact, GSTP is an enzyme actively involved in the response to oxidative stress and we have already shown that it plays a role in the context of MPTP-induced lesions (Castro-Caldas *et al*., 2012). To assess the effects of *Gstp1 and Gstp2* genes deletion, and MPTP and/or TUDCA treatment in the expression levels of the three UPR branches, Western blot assays for wt and GSTP KO cortex samples were conducted.

Results from Figure 5 show that in the GSTP KO mice cortex ATF6α protein levels are significantly reduced when compared to their wt counterparts in all of the conditions studied, except for control samples and in samples from mice treated with TUDCA and sacrificed 3 h after MPTP administration. Although we did not observe any effect of MPTP treatment on the expression levels of ATF6α in wt mice, when GSTP KO mice were treated with MPTP, the expression levels of ATF6α were significantly decreased. In order to evaluate the putative neuroprotective effects of TUDCA, a group of animals was treated with TUDCA. Similarly to what happened in MPTP-treated wt mice, TUDCA administration for three consecutive days had no effect in the expression levels of ATF6α. However, TUDCA led to a decrease in ATF6α expression levels in GSTP KO mice. Considering the wt mice, pre-treatment with TUDCA for three consecutive days prior to administration of MPTP for 6 h increased the expression levels of ATF6α. In the case of GSTP KO mice, the same treatment

schedule with TUDCA and MPTP decreased ATF6α expression levels at the 3 h time-point, when compared to the expression levels observed in control samples.

Concerning the phosphorylated levels of PERK (p-PERK), we observed a decrease in the levels of this protein in the control samples of GSTP KO mice when compared to the corresponding wt control samples (Fig. 6). Treatment with MPTP resulted in different outcomes; it induced a significant decrease in the levels of p-PERK in wt mice, while it did not produce any change in GSTP KO mice. In the samples obtained from mice treated with TUDCA we observed a decrease in p-PERK levels in wt mice when compared to the corresponding controls. Furthermore, pre-treatment with TUDCA followed by a single MPTP administration for 3 or 6 h increased the expression levels of p-PERK in the wt mice, attaining the highest levels 6 h after MPTP administration, when compared to the control samples. Regarding the GSTP KO mice, pre-treatment with TUDCA had no significant effect at the 3 h time-point but induced a significant increase in p-PERK levels at the 6 h time point, when compared to the respective control samples.

In the case of IRE1α, there is a significant increase in the expression levels of this ER stress mediator in the GSTP KO control samples when compared to the wt (Fig. 7). Curiously, the MPTP and TUDCA treatment had no significant effect on the expression levels of IRE1α in wt mice, but led to a significant decrease in the expression of IRE1α in GSTP KO mice. This decrease in IREα expression was also observed in GSTP KO mice pre-treated with TUDCA and sacrificed after MPTP administration. In fact, there was a significant decrease in IRE1α protein expression when compared to control samples that was similar at both 3 h and 6 h time-points. In wt samples, however, pre-treatment with TUDCA had a different effect; an increase in the expression levels of IRE1α was observed at the 6 h time-point in comparison with control samples and no significant changes were seen at the 3 h time-point

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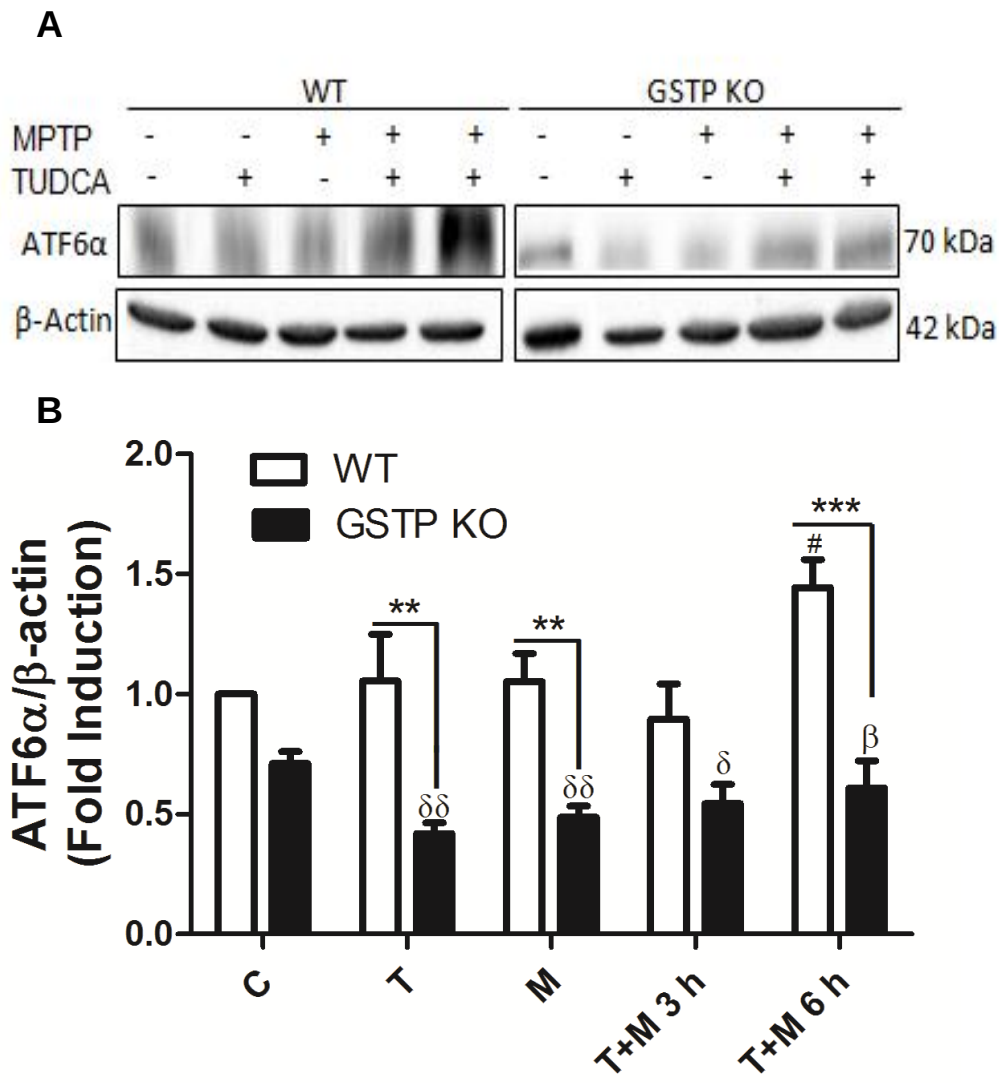

Figure 5 - ATF6α expression levels in the brain cortex in response to treatment with TUDCA, MPTP or TUDCA + MPTP. C57/BL6 wild type and GSTP KO mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. (**A**) ATF6α levels were determined by Western blot analysis, using a mouse anti-ATF6 antibody. Analysis of β-actin was done in parallel as a loading control. The immunoblots shown are representative of three independent experiments. (**B**) The ATF6α levels in wt control samples were arbitrarily set as 1 and the relative levels in MPTP, TUDCA and TUDCA + MPTP samples were calculated and plotted as a fold induction over control. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni post-hoc test where # $p < 0.05$ relative to wild type control; δ $p < 0.05$; δδ $p < 0.01$ relative to GSTP KO control; β $p < 0.05$ relative to TUDCA GSTP KO; ** $p < 0.01$; *** $p < 0.001$ wild-type vs. corresponding GSTP KO.

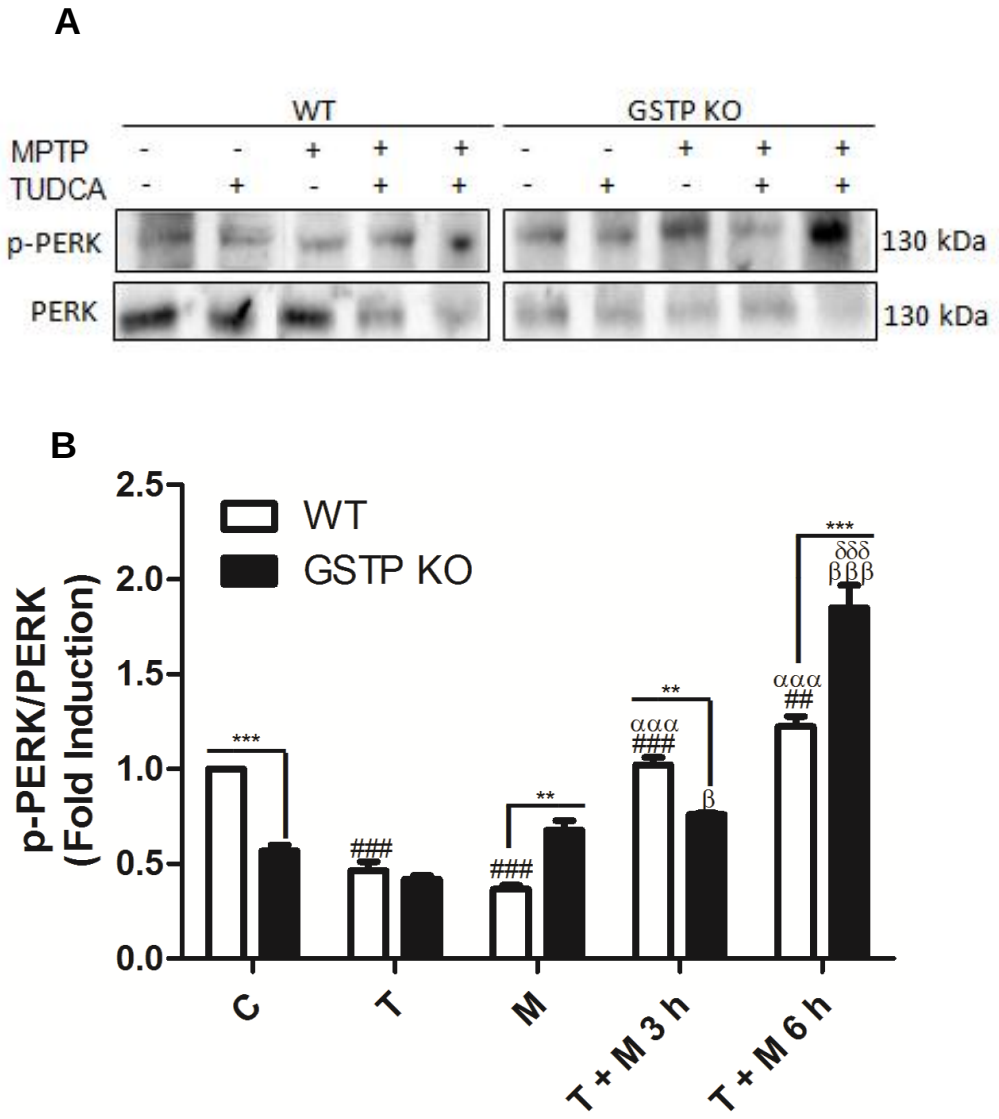

Figure 6 – p-PERK expression levels in the brain cortex in response to treatment with TUDCA, MPTP or TUDCA + MPTP. C57/BL6 wild type and GSTP KO mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. (**A**) p-PERK levels were determined by Western blot analysis, using a rabbit anti-p-PERK antibody. Analysis of PERK was done in parallel as a loading control. The immunoblots shown are representative of three independent experiments. (**B**) The p-PERK levels in wt control samples were arbitrarily set as 1 and the relative levels in MPTP, TUDCA and TUDCA + MPTP samples were calculated and plotted as a fold induction over control. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni post-hoc test where ### $p < 0.001$ relative to wild type control; ; δδδ $p< 0.001$ relative to GSTP KO Control; ; ααα $p < 0.001$ relative to TUDCA wild type; βββ $p < 0.001$ relative to TUDCA GSTP KO; ** $p < 0.01$; *** $p < 0.001$ wild-type vs. corresponding GSTP KO.

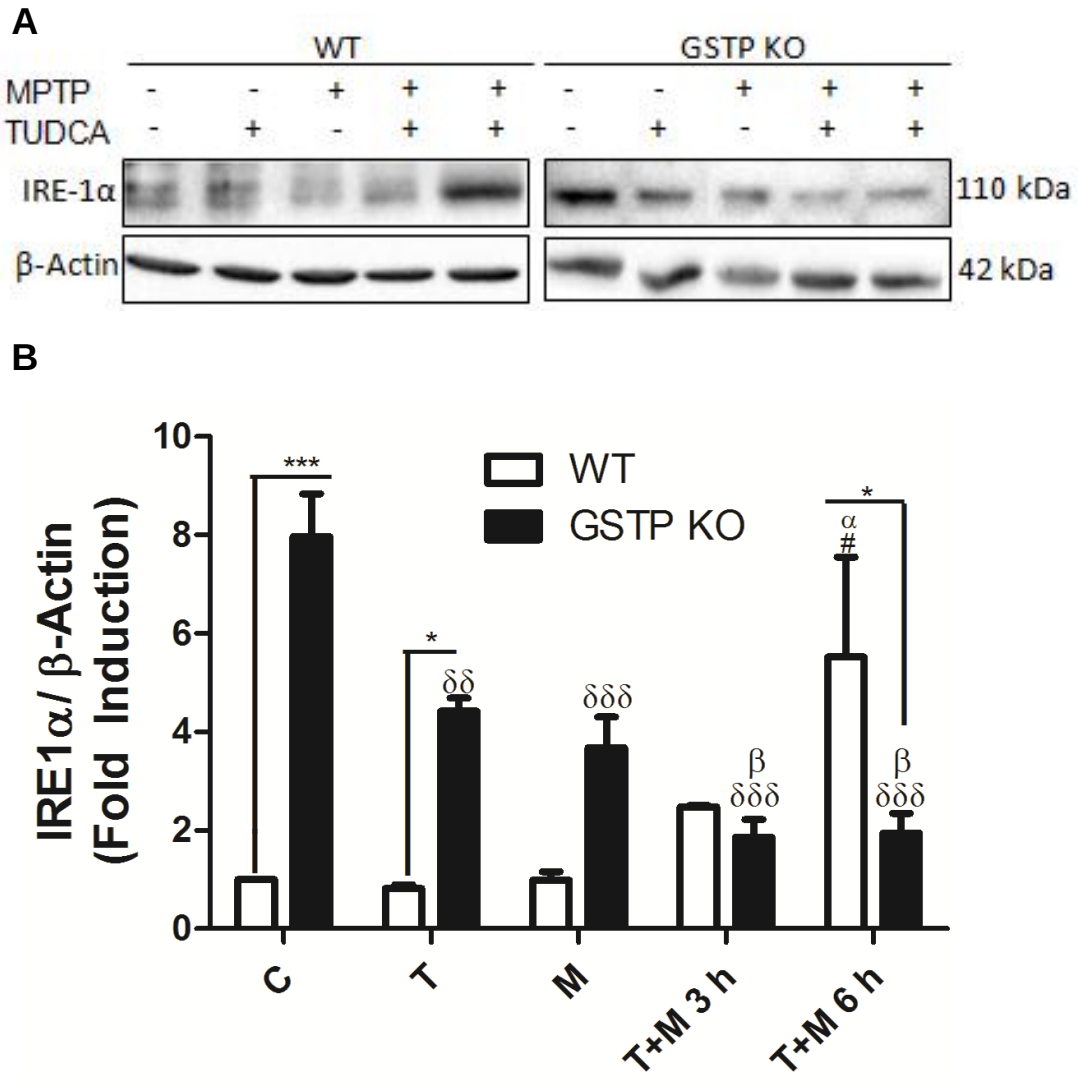

Figure 7 – IRE1α expression levels in the brain cortex in response to treatment with TUDCA, MPTP or TUDCA + MPTP. C57/BL6 wild type and GSTP KO mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. (**A**) IRE1α levels were determined by Western blot analysis, using a rabbit anti-IRE1α antibody. Analysis of β-actin was done in parallel as a loading control. The immunoblots shown are representative of three independent experiments. (**B**) The IRE1α levels in wt control samples were arbitrarily set as 1 and the relative levels in MPTP, TUDCA and TUDCA + MPTP samples were calculated and plotted as a fold induction over control. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni post-hoc test where # $p < 0.05$ relative to wild type control; δδ $p < 0.01$; δδδ $p < 0.001$ relative to GSTP KO Control; α $p < 0.05$ relative to TUDCA wild type; β $p < 0.05$ relative to TUDCA GSTP KO; * $p < 0.05$; *** $p < 0.001$ wild-type vs. corresponding GSTP KO.

2. The expression levels of downstream effectors of the UPR pathways are altered in GSTP KO mice

After measuring the expression levels of the three mediators of the UPR, we sought to analyze the expression levels of the downstream effectors of these pathways. Even though changes were observed in the protein expression levels of ATF6α, IRE1α and PERK, this does not necessarily mean that these pathways are activated. By analyzing the expression levels of p-eIF2α, a transcription factor phosphorylated by PERK, and the expression levels of CHOP, a transcription factor modulated by both the PERK and ATF6α branches of the UPR, we can perceive if the UPR mediators are indeed activating its respective effectors. With this purpose Western blot assays for wt and GSTP KO cortex samples were conducted to assess the protein expression levels of p-eIF2α and CHOP.

In the case of p-eIF2α, (Fig. 8), the only significant changes were seen in GSTP KO mice treated with TUDCA for three consecutive days followed by single MPTP administration and sacrificed 6 hours after (T+M 6h), which showed an increase of expression when compared to their corresponding control samples. This result is similar to the previously described for p-PERK expression levels in the same analyzed samples. We also observed that in the wt and GSTP KO mice treated with TUDCA for three consecutive days the p-eIF2α expression levels were significantly different, although no significant changes were detected when comparing with the respective control samples.

Regarding the expression levels of CHOP, we found no significant changes in the expression levels of this protein. We observed that in the wt and GSTP KO mice pre-treated with TUDCA and sacrificed after 6 h of MPTP administration the CHOP expression levels are significantly different but these values show no significant changes when comparing with the control samples (Fig. 9).

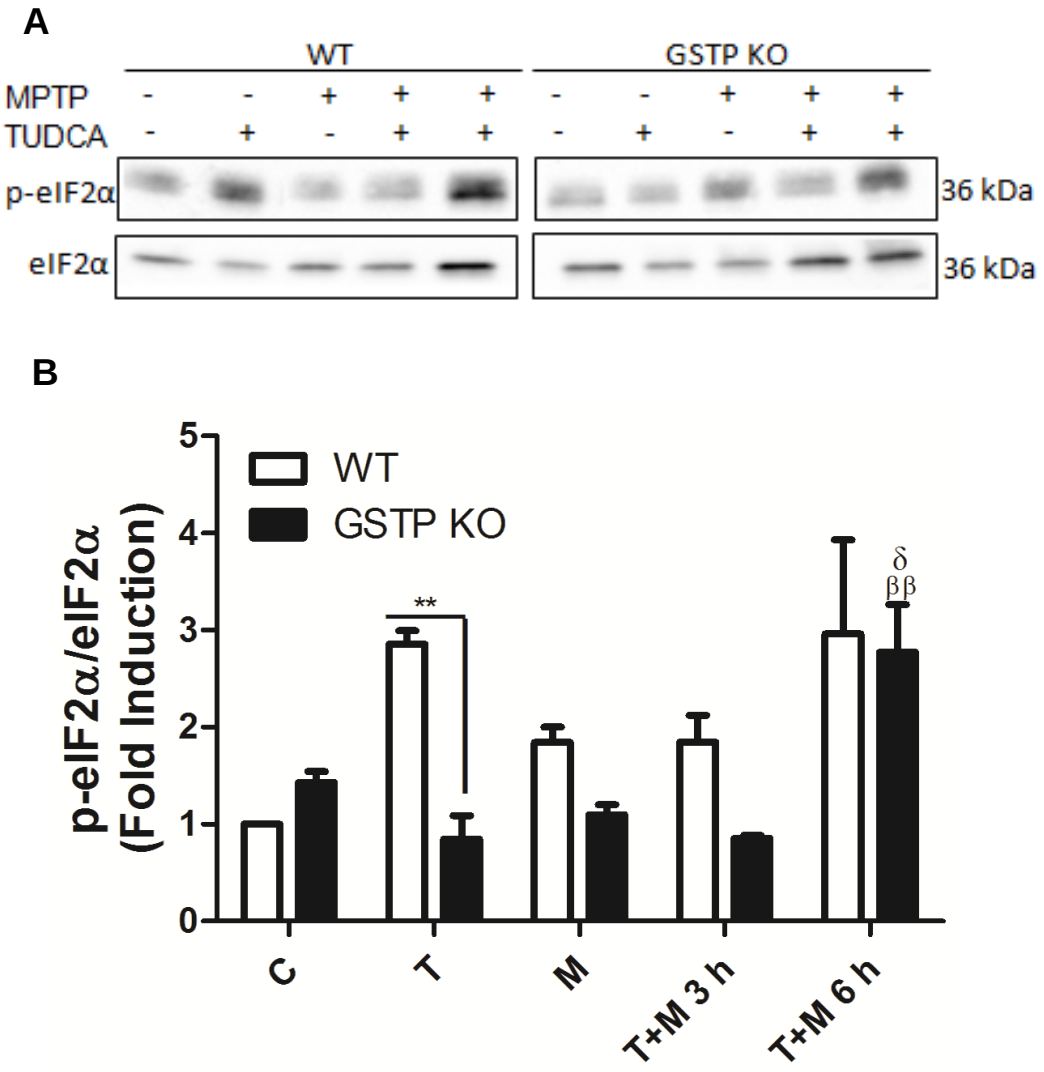

Figure 8 – p-eIF2α expression levels in the brain cortex in response to treatment with TUDCA, MPTP or TUDCA + MPTP. C57/BL6 wild type and GSTP KO mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. (**A**) p-eIF2α levels were determined by Western blot analysis, using a rabbit anti-p-eIF2α antibody. Analysis of eIF2α was done in parallel as a loading control. The immunoblots shown are representative of three independent experiments. (**B**) The p-eIF2α levels in wt control samples were arbitrarily set as 1 and the relative levels in MPTP, TUDCA and TUDCA + MPTP samples were calculated and plotted as a fold induction over control. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni post-hoc test where δ $p< 0.05$ relative to GSTP KO Control; ββ $p < 0.01$ relative to TUDCA GSTP KO; ** $p < 0.01$ wild-type vs. corresponding GSTP KO. GSTP KO; * $p < 0.05$; *** $p < 0.001$ wild-type vs. corresponding GSTP KO.

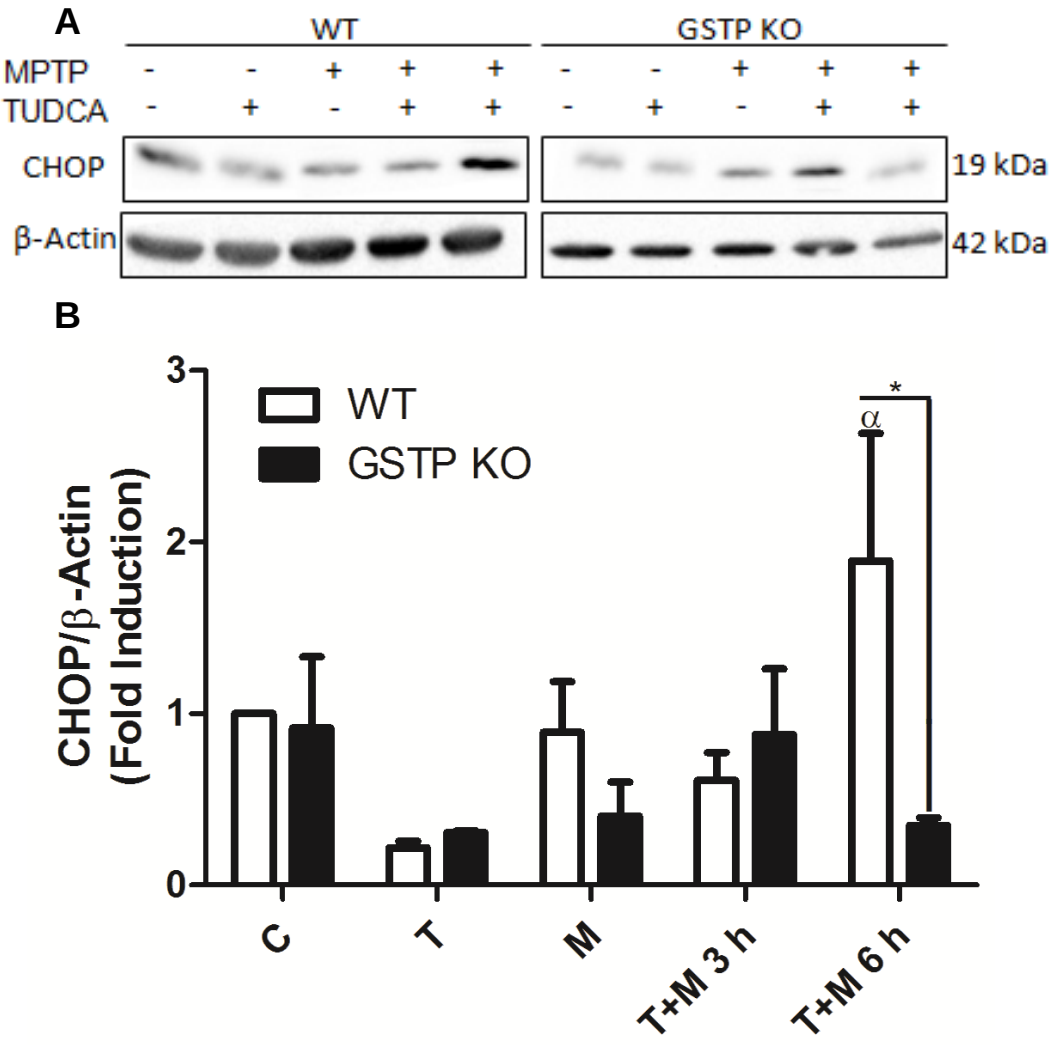

Figure 9 – CHOP expression levels mice cortex in response to treatment with TUDCA, MPTP or TUDCA + MPTP. C57/BL6 wild type and GSTP KO mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. (**A**) CHOP levels were determined by Western blot analysis, using a rabbit anti-CHOP antibody. Analysis of β-actin was done in parallel as a loading control. The immunoblots shown are representative of three independent experiments. (**B**) The CHOP levels in wt control samples were arbitrarily set as 1 and the relative levels in MPTP, TUDCA and TUDCA + MPTP samples were calculated and plotted as a fold induction over control. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni post-hoc test where α $p < 0.05$ relative to TUDCA wild type; β $p < 0.05$ relative to TUDCA GSTP KO; * $p< 0.05$ wild-type vs. corresponding GSTP KO.

3. Nrf2 expression levels are increased in wild type mice treated with MPTP

The trigger for dysfunctional protein metabolism, in sporadic PD, may be oxidative stress through damage caused by ROS. One potential defence against the toxicity of ROS is the up-regulation of phase II detoxification enzymes, namely GSTP, by the Nrf2 transcription factor. Moreover, this transcription factor can also be activated through a pathway dependent of the UPR mediator PERK. Furthermore, preliminary results from our group have shown that MPTP-induced dopaminergic neuronal degeneration is an earlier event when comparing GSTP null versus wt mice, suggestive of a protective role for GSTP (Castro-Caldas *et al*., 2012).

So afterwards we evaluated the Nrf2 protein levels in the cortex of both wt and GSTP KO mice by Western blot assay using a specific antibody.

No significant differences were observed in the expression levels of Nrf2 between wt and GSTP KO samples, except for the MPTP treatment (Fig. 10). In fact, treatment with MPTP results in increased expression levels of Nrf2 in the wt mice cortex samples.

To further analyze the expression of Nrf2 in the experimental conditions already described and in order to evaluate its sub-cellular distribution in the mice brain cortex, immunohistochemistry assays were conducted using coronal sections of wt mice brain (Bregma 0.38). Results presented in Figure 11 (panel c) show that treatment with MPTP promotes the nuclear translocation of Nrf2 as demonstrated by the co-localization of the green fluorescence for Nrf2 antibody with the blue fluorescence for the nuclear marker, Hoechst. As shown in Figure 11 (panel b), TUDCA treatment results in an increase in total Nrf2 levels which appears to be widespread throughout the cell (Fig.11 – panel b, insert). We also observed that Nrf2 expression is also increased when MPTP was administered following TUDCA for 3 consecutive days, with higher levels detected at the 3 h time point rather than the 6 h time point (Fig. 11- e and 11- d, respectively).

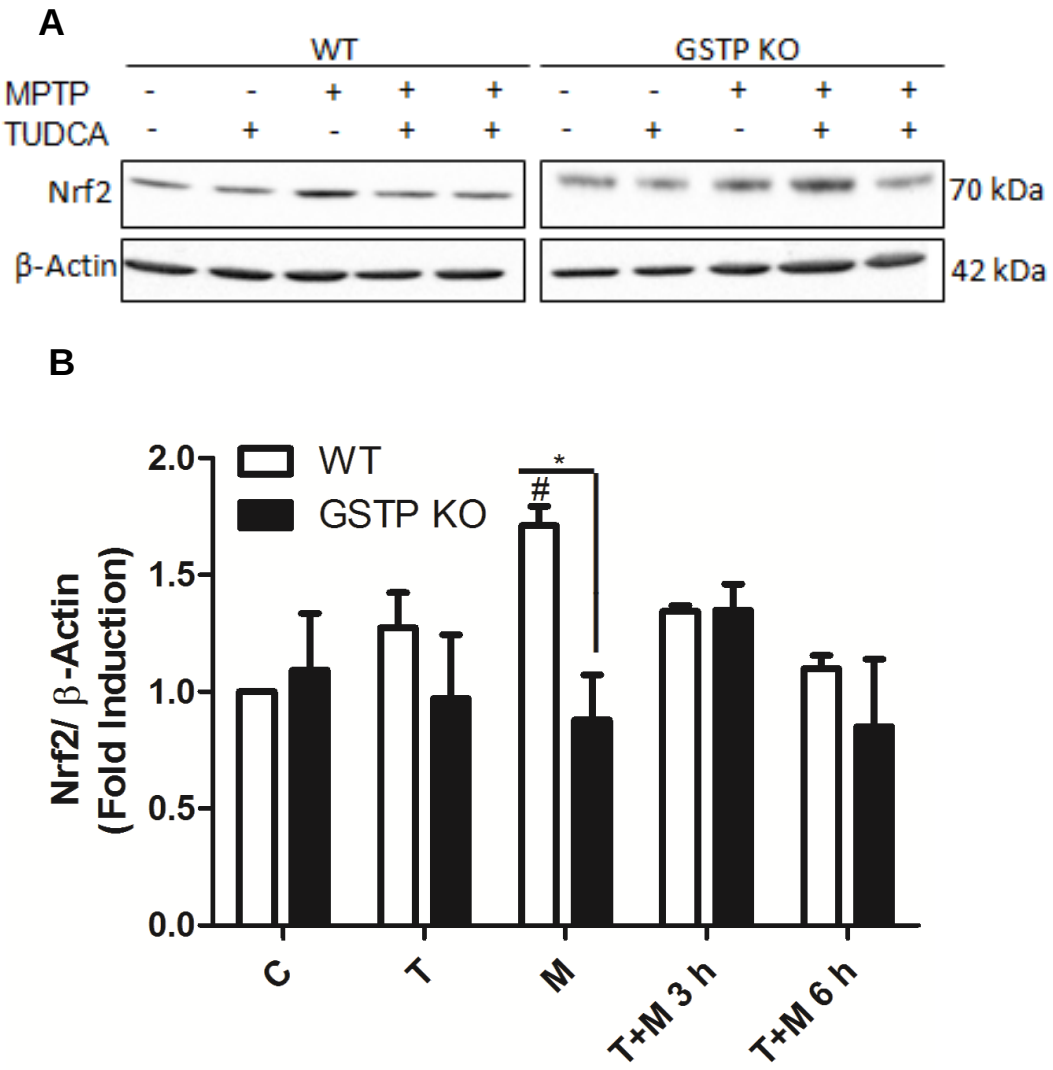

Figure 10 – Nrf2 expression levels in the brain cortex in response to treatment with TUDCA, MPTP or TUDCA + MPTP. C57/BL6 wild type and GSTP KO mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. (**A**) Nrf2 levels were determined by Western blot analysis, using a mouse anti-Nrf2 antibody. Analysis of β-actin was done in parallel as a loading control. The immunoblots shown are representative of three independent experiments. (**B**) The Nrf2 levels in wt control samples were arbitrarily set as 1 and the relative levels in MPTP, TUDCA and TUDCA + MPTP samples were calculated and plotted as a fold induction over control. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni post-hoc test where # $p < 0.05$ relative to wild type control; * $p < 0.05$; wild-type vs. corresponding GSTP KO.

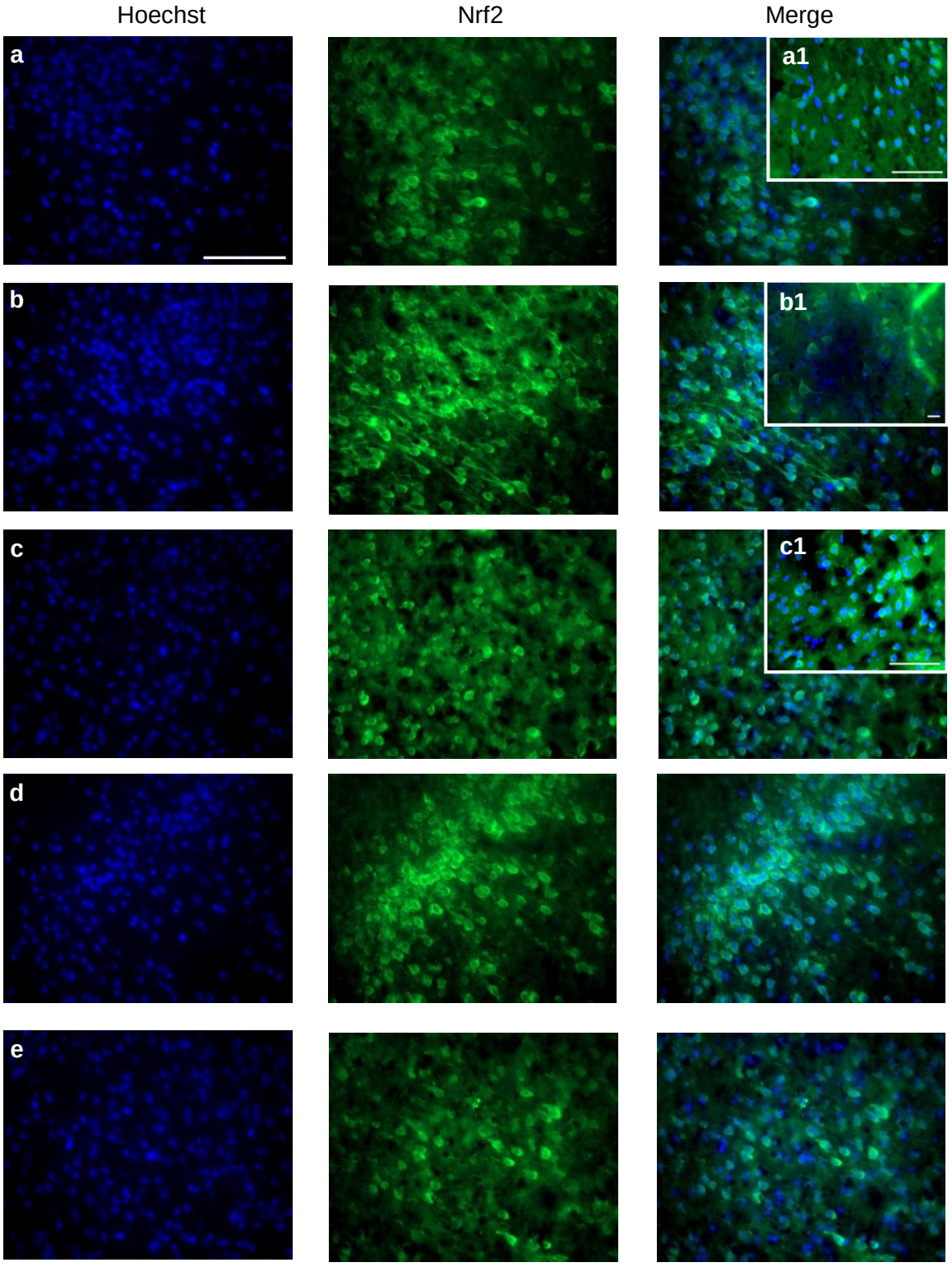

Figure 11 – Nrf2 sub-cellular distribution in the brain cortex of C57/BL6 wild type mice in response to treatment with TUDCA, MPTP or TUDCA + MPTP. Mice were i.p. injected with saline (control, C), TUDCA (T; 50 mg/kg), MPTP (M; 40 mg/kg) or TUDCA + MPTP and sacrificed 3h (T+M 3h) or 6h (T+M 6h) after MPTP administration. Coronal sections at the level of the cerebral cortex (Bregma 0.38) from C57BL/6 wild-type mice were immune stained for Nrf2 protein (green). Hoechst (blue) was used as nuclear marker. Representative microphotographs from saline (**a** and **a1**), TUDCA (**b and b1**), MPTP (**c** and **c1**) and T+M 3h (**d**) or T+M 6h (**e**) are shown. Scale bar =100 µm; insert = 100 µm

IV. DISCUSSION

In this work we aimed to evaluate the expression levels of ER stress markers in C57/BL6 wt and GSTP KO mice brain using a sub-acute MPTP model of PD. Throughout this project both wt and Gstp null mice were used in order to clarify the potentially neuroprotective role of the GST isoform pi, GSTP, by studying ER stress responses in the context of MPTP-induced brain lesions. Moreover, in parallel, TUDCA was used as a chemical chaperone already described as an ER stress reducer.

In the following section we will discuss our results focusing on: (a) the effects of MPTP administration, (b) the effects of GSTP null mouse genotype, and (c) the effects of TUDCA treatment.

Although MPTP has been described as having a regional specificity towards dopaminergic neurons in the striatum (Dauer and Przedborski, 2003), there are also reports of DA reduction in the cortex, as well as biochemical alterations in all catecholaminergic neurons resulting from MPTP neurotoxicity (Hallman *et al*., 1984; Wallace *et al*., 1984).

Concerning the UPR modulation, it has been shown that ATF6α and PERK/eIF2α/ATF4 pathways are activated in mice nigrostriatal dopaminergic neurons upon treatment with MPTP (Hashida *et al*., 2012). However, our results show no significant differences in ATF6α expression levels in the cortex of wt mice following MPTP administration and p-PERK expression levels are actually decreased when compared to controls. We also found that in response to MPTP treatment, the protein levels of CHOP, a downstream effector of ATF6α, are not significantly different from the controls. These results are in contradiction with what was previously described. In fact, it has been shown that MPTP induces CHOP expression both *in vitro* (Holtz and O'Malley, 2003) and *in vivo* (Silva *et al*., 2005) in the SN of mice treated with this neurotoxin. Our work focused on the effects of MPTP in the brain cortex and, to our knowledge, there are not any reports concerning CHOP expression in this brain region in the context of MPTP lesions. Given the fact that MPTP may have a more pronounced and specific effect in the SN, it is possible that in the cortex it may trigger a mild ER stress response, in which sub-lethal levels of ER stress selectively engage adaptive UPR signaling events, a response that may involve the expression of XBP1s but not CHOP (Fouillet *et al*., 2012).

Looking at the IRE1α branch of the UPR, our results suggest that this pathway may not be involved in the ER-stress response to MPTP exposure in the cortex of wt mice, as IRE1α expression levels in the neurotoxin-treated mice are not increased when compared to their controls. These results are in accordance with previously described work, showing that IRE1α is not activated in the event of MPTP-induced lesions (Sado *et al*., 2009).

In the light of these results, we can speculate that in this model of single acute MPTP administration there is no increase in ER stress markers. This may be explained through several reasons: the first one, as previously mentioned, is that in this model, MPTP may only trigger a mild ER stress response. Secondly, the time-point used for MPTP administration (mice were sacrificed 6 h after single MPTP administration) may not be the most appropriate. Previously our group has reported that 6 h after MPTP administration p-JNK control levels are restored (Castro-Caldas *et al*., 2012). But since we are looking at ER stress markers, this time-point may not be adequate. Thirdly, the number of samples analyzed was not ideal and some of these samples present a high variability, which makes definite results harder to achieve.

Further studies are needed at different time-points following MPTP administration and a larger number of samples should be analyzed.

Our Western blot results also revealed that Nrf2 expression is increased in MPTP-treated wt mice when compared to controls. In parallel, higher nuclear translocation was detected in the immunohistochemistry assay, suggestive of an activation of Nrf2 in the cortex of MPTP-treated mice. These results are in accordance to the literature, as Nrf2 regulates the adaptive response to oxidants and electrophiles (Ma, 2008). Moreover, our results are also supported by reports using the MPTP mouse model in which the knockout of *Nrf2* in mice increased the sensitivity to MPTP (Chen *et al*., 2009). Also, others have shown that the knockout of *Nrf2* in mice increases the susceptibility to a broad range of chemical toxicity and disease conditions associated with oxidative pathology (Chan *et al*., 2001; Motohashi and Yamamoto, 2004; Walters *et al*., 2008). According to our results, it seems that Nrf2 is actively involved in the response to oxidative stress and in the response to MPTP-induced oxidative stress in particular, probably contributing to cell survival and redox homeostasis.

We also sought to evaluate the potential neuroprotective effect of GSTP in the context of ER stress induced by MPTP-induced oxidative stress. The most interesting results obtained concern the AFT6α and IRE1α proteins. The protein expression analyses of ATF6α showed that there is a decrease in the expression levels of this protein in GSTP KO mice when compared to their wt counterparts in most of the conditions studied. We may speculate that in the absence of GSTP, ATF6α is down-regulated. This hypothesis may be explained by a possible up-regulation of ATF2, a transcription factor that can bind to GSTP through protein-protein interactions, which results in its inhibition (Thévenin *et al*., 2011). ATF2 has been shown to be connected in a negatively acting feedback loop to the MAPK p38 through the activation of p38-specific phosphatases (Breitwieser *et al*., 2007). The p38 kinase is an enzyme that promotes the phosphorylation of ATF6α in the event of ER stress, inducing its transcriptional activity and enhancing ATF6α ability to transactivate certain genes (Thuerauf *et al*., 1998; Luo and Lee, 2002; Egawa *et al*., 2011). It would have been interesting to analyze if the deletion of *Gstp1/2* results in increased ATF2 expression levels, which in turn would negatively regulate p38, decreasing its expression, leading to a decreased activation of ATF6α.

Regarding the IRE1α pathway of the UPR, *Gstp1/2* deletion has the opposite effect when compared with ATF6α. IRE1α expression levels in control and MPTP-treated samples are higher than their wt counterparts, suggesting an up-regulation of this pathway. One possible explanation for this putative up-regulation may be trough an increased TRAF2 activation. It has been shown that over-expression of GSTP attenuates TRAF2-ASK1 auto-phosphorylation by suppressing the interaction between TRAF2 and ASK1, and silencing of GSTP results in an increase in TRAF2-ASK1 association (Wu *et al*., 2006). It has also been shown that the cytoplasmic domain of IRE1α bounds TRAF2, which in turn activates the JNK pathway (Urano *et al*., 2000). We may speculate that TRAF2-ASK1 increased association due to *Gstp1/2* deletion may prompt an increase in IRE1α expression levels due to an unknown crosstalk mechanism, meaning that GSTP probably may regulate IRE1α expression through an indirect interaction with this UPR mediator.

In conclusion, we speculate that GSTP may have a neuroprotective effect concerning the ATF6α pathway of the UPR, as GSTP KO mice present a decreased expression of this protein. In the case of IRE1α, we speculate that overexpression of GSTP may result in a decreased expression of this protein.

In this work we also wanted to evaluate the possible neuroprotective role of GSTP using the MPTP mouse model of PD. The most prominent effect of *Gstp1/2* deletion was observed in the MPTP-treated samples, in which Nrf2 expression levels have a significant decrease when compared to their wt counterparts. This may result in a deficient response to oxidative stress caused by MPTP, which may prompt an increase in apoptosis. It has been shown that Keap1, which physically interacts with Nrf2 and promotes its ubiquitination driving it for proteosomal degradation (Kobayashi *et al*., 2004), is modified by S-glutathionylation. This post-translational modification might result in the dissociation of Keap1 from Nrf2, promoting Nrf2 activation and the consequent synthesis of proteins involved in the antioxidant response (Zhang *et al*., 2010). Previous reports have shown that GSTP is actively involved in S-glutathionylation following nitrosative and oxidative stress (Townsend *et al*., 2009). Even though GSTP KO mice samples do not present significant changes from their respective controls, the results point towards a tendency of lower expression levels of Nrf2 in GSTP KO mice samples treated with MPTP when comparing to their respective controls. We may speculate that GSTP may be involved in Keap1 S-glutathionylation and in the absence of GSTP reactive Keap1 cysteines residues are not glutathionylated, which may result in increased ubiquitination of Nrf2 and consequent proteosomal degradation.

The final goal of this work was to assess the potential therapeutic value of TUDCA both in the context of MPTP-induced oxidative stress and consequent ER stress. Previously, our group has reported that TUDCA can prevent MPTP-induced cell death in dopaminergic neurons (Castro-Caldas *et al*., 2012).

Our results regarding the analysis of ATF6α protein expression show that TUDCA treatment had no significant changes the expression of this ER stress mediator in the brain cortex of wt mice while decreasing its expression in TUDCA-treated GSTP KO mice when compared to control samples. The results

for wt mice are supported by reports that show that TUDCA does not have any effect in the expression levels of ATF6α (Hua *et al*., 2010). When TUDCA was administered before MPTP exposure, an increase of expression is seen at the 6 h time-point. However, in GSTP KO mice, pre-treatment with TUDCA promotes a decrease in the expression of ATF6α at the 3 h time-point while showing no significant changes at the 6 h time-point when comparing to control samples, suggesting that *Gstp1/2* deletion may result in a more prominent decrease in the expression levels of ATF6α. We speculate that the TUDCA pre-treatment coupled with inhibition of GSTP may have a more significant effect in modulating ATF6α expression levels at earlier stages of MPTP administration.

Concerning the ATF6α downstream mediator CHOP, even though we could not detect any significant difference, in samples from TUDCA-treated mice there is a tendency to a reduction in CHOP levels when comparing to their respective controls, an effect that has been previously described (Malo *et al*., 2010; Gao *et al*., 2012).

In the case of IRE1α, treatment with TUDCA had no significant effect in the expression levels of this protein in the cortex of wt mice, while showing a significant

decrease in GSTP KO mice. This data is in contrast to previous reports that show that TUDCA promotes the suppression of IRE1α and consequent JNK activation (Ozcan *et al*., 2006). Furthermore, pre-treatment with TUDCA appears to have the opposite effect on wt mice, showing an increase of the expression levels of IRE1α at the 6 h time point. This could mean that in the event of MPTP-induced neurotoxicity, TUDCA promotes the increase of IRE1α expression levels as a compensatory mechanism, which then may induce expression of chaperones and ERAD components, unloading ER burden.

Looking at the PERK pathway, treatment with TUDCA reduced p-PERK expression levels in wt mice when compared to the respective controls, an effect that has been previously described (Ozcan *et al*., 2006). However, other reports have shown that TUDCA promotes the increase of p-PERK expression levels (Seyhun *et al*., 2011; Gani *et al*., 2015) so it is not exactly clear yet how TUDCA exerts its effects in the modulation of p-PERK expression levels. Pre-treatment with TUDCA increased the phosphorylation levels of PERK in wt mice

more prominently at the 6 h time point, suggesting that in the event of MPTP-induced neurotoxicity, TUDCA may promote the expression of p-PERK, with a more prominent effect in later stages following the insult.

Although speculative, we may conclude that TUDCA modulates ER stress in early stages of MPTP-induced oxidative stress while promoting the expression of mediators of the UPR pathways in later stages.

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